THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY

VOL. IX APRIL, 1928 NO. 2

THE MODIFICATION OF SCARLATINAL TOXIN BY FORMALDEHYDE.

J. SMITH, M.D., D.Sc., D.P.H.

From the City Hospital Laboratory, Aberdeen.

Received for publication February 7th, 1928.

THE experience derived from the immunization of susceptible individuals (Kinloch, Smith and Taylor, 1927) with amounts of scarlatinal toxin ranging from 500 to 3000 skin test doses, has shown that both the local and general reactions produced by this substance are occasionally rather severe. Accordingly attention has been directed to the preparation of a toxoid or anatoxin in the hope that the toxic effects of the immunizing agent would be partly or wholly eliminated, and that by being able to give larger doses of antigen, a greater degree of antitoxic immunity might be produced.

Only two published reports on the modification of scarlatinal toxin by the action of formaldehyde have been observed. Zoeller (1925) produced scarlatinal anatoxin by adding 0·5% formalin to scarlatinal toxin and placing the mixture in the incubator at 37°C. for 24 days. He claimed that by this means much larger doses of toxin (1500 to 2000 Dick unit doses) could be tolerated at the first injection. Sparrow and Celarek (1927) prepared anatoxin by adding 0·5% formalin to a scarlatinal toxin containing 20,000 Dick units per c.c., and incubating the mixture at 38° C. for 6 weeks. They found that susceptible individuals could tolerate an injection of 2 c.c. of this modified toxin, and that in a series of cases immunized with a single injection of 2 c.c., 80% became negative to the Dick test dose of toxin a month after inoculation. A further immunizing dose was given to those remaining positive, and when re-tested two months later only 6% of 143 cases remained refractory to immunization.

ACTION OF FORMALDEHYDE ON SCARLATINAL TOXIN.

The toxin used in this investigation was prepared by inoculating two flasks each containing 1500 c.c. of Hartley's modification of Douglas's trypsin broth with 10 c.c. of a 24-hour broth culture of the Dochez strain. The cultures were then incubated for 48 hours at 37°C. and passed through a sterile Seitz

filtering apparatus. The toxin was roughly standardized on a series of 25 individuals by comparing the skin reactions produced by various dilutions with those produced by the Dick test toxin, and it was found that 0·2 c.c. of 1 in 2000 dilution produced reactions comparable to those produced by the standard toxin so that the undiluted toxin contained 10,000 skin test doses per c.c.

The filtrate was distributed into bottles, and various concentrations of formalin (38·76% formaldehyde) were added.

1. 250 c.c. toxin containing 2% formalin
2. 1000 „ „ „ 1% „
3. 1000 „ „ „ 0·5% „
4. 100 „ „ without formalin

} Placed in incubator at 37°C.

5. 250 „ „ „ „ —stored in refrigerator.

After incubation at 37°C. the action of the formaldehyde on the toxin was studied by ascertaining whether various dilutions of the several mixtures were capable of inducing skin reactions in susceptible individuals. The skin reactions noted in the following pages were classified as follows:

No inflamed area . . . . = 0
Inflamed area 1 cm. in diameter = +
„ „ 1–2 „ „ = ++
„ „ 2–3 „ „ = +++
„ „ over 3 cm. „ = ++++

The first skin tests were made after an incubation period of six weeks, and the results of these and subsequent tests are recorded in Table I. The first series of tests showed a marked decrease in strength of the toxin containing 2% formalin and a slight decrease in the 1% and 0·5% formalinized toxins and also of the control toxin, to which no formalin or other preservative had been added. A further series of tests using the toxin containing 2% formalin, diluted to 1 in 50 and 1 in 10, showed that in these dilutions the toxin was still capable of producing marked reactions.

TABLE I.—*Results of Skin Tests.*

First Test after an Incubation Period of 6 weeks.

Case.	Dick test toxin.	Formalinized toxin. 2%/2000	Formalinized toxin. 1%/2000	Formalinized toxin. 0·5%/2000	Toxin without formalin. /2000	Control heated toxin. /2000
First series 1	++++	0	++	+++	+++	0
First series 2	++	0	+	+	+	0
First series 3	++++	0	++	+++	+++	0

Case.	Dick test toxin.	Formalinized toxin. 2%/50	Formalinized toxin. 2%/10	Control heated toxin. /10
Second series 1	+	+++	+++	0
Second series 2	++	++++	++++	0
Second series 3	+	+++	++++	0

TABLE I—*continued.*

Second Test after a total Incubation Period of 9 weeks.

Case.	Dick test toxin.	Formalinized toxin. 2% / 50	Formalinized toxin. 2% / 10	Control heated toxin. / 2000
1	++++	++++	++++	0
2	++++	++++	++++	0
3	0	++	++++	0

Third Test after a total Incubation Period of 13 weeks.

	Case.	Dick test toxin.	Formalinized toxin. 2% / 50	Formalinized toxin. 1% / 10	Formalinized toxin. 0·5% / 50	Incubated control toxin. / 1000	Control heated toxin. / 50
First series	1	++++	0	+	+++	++++	0
	2	++++	0	+	+++	+++	0
	3	++	0	0	0	++	0

	Case.	Dick test toxin.	Formalinized toxin. 2% / 10	Control heated toxin. / 10
Second series	1	+++	++	0
	2	+	0	0
	3	++	+	0

Fourth Test after a total Incubation Period of 16 weeks.

Case.	Dick test toxin.	Formalinized toxin. 2% / 50	Formalinized toxin. 2% / 10	Formalinized toxin. 1% / 50	Formalinized toxin. 0·5% / 50	Control heated toxin. / 10
1	+++	0	0	+	+++	0
2	+++	0	0	+	++	0
3	+++	0	0	+	++	0

Fifth Test after a total Incubation Period of 29 weeks.

	Case.	Dick test toxin.	Formalinized toxin. 1% / 50	Formalinized toxin. 0·5% / 50	Control heated toxin. / 50
First series	1	++++	0	++++	0
	2	+++	0	+++	0
	3	+++	0	+++	0
	4	+++	0	+++	0

	Case.	Dick test toxin.	Formalinized toxin. 1% / 10	Control heated toxin. / 10
Second series	1	+++	+	0
	2	+	0	0
	3	++	+	0
	4	++++	++	0

Sixth Test after a total Incubation Period of 50 weeks.

Case.	Dick test toxin.	Toxin incubated without formalin. Toxin. / 2000	Formalinized toxin. 0.5% / 100	Formalinized toxin. 0·5% / 10	Control heated toxin. / 10
1	++++	+++	0	++	0
2	++	++	0	0	0
3	++++	+++	0	++	0
4	++++	+++	0	+++	0
5	++++	+++	0	++	0
6	++++	++++	0	+++	0

After an incubation period of 9 weeks the next series of tests did not show any further marked decrease in activity, but after an incubation period of 13 weeks the skin tests (third test) showed that the toxin containing 2% formalin was only capable of inducing slight reactions in a dilution of 1 in 10, while the toxin containing 1% formalin induced slight reactions with a 1 in 50 dilution, and the 0·5% formalinized toxin induced marked reactions when used in a dilution of 1 in 50.

After an incubation period of 16 weeks (fourth test), the 2% formalinized toxin produced no reaction when diluted 1 in 10. Slight reactions were produced by the toxins containing 1% formalin when diluted 1 in 50, and marked reactions were produced by the toxin containing 0·5% formalin when used in a similar dilution.

The fifth series of tests made after an incubation period of 29 weeks showed that the toxin containing 1% formalin induced only feeble reactions in a dilution of 1 in 10, and therefore 1 c.c. of this toxin now contained much less than 50 skin test doses of active toxin per c.c. At this time a 1 in 50 dilution of the 0·5 % formalinized toxin still produced reactions similar in size to those induced by the Dick test dose.

The sixth series of tests made after an interval of 50 weeks showed that the toxin to which 0·5% formalin had been added produced reactions, in a dilution of 1 in 10, markedly less in degree than those produced by the Dick test toxin. On the other hand, the sample of the toxin which did not contain formalin or other preservative and which had been kept in the incubator over the whole period was still capable, in a dilution of 1 in 2000, of inducing skin reactions which were only slightly less marked than those induced by the Dick test dose.

THE IMMUNIZING VALUE OF VARIOUS TOXOIDS.

Unfortunately the immunizing value of scarlatinal antigens cannot be determined with any degree of precision by animal tests. When, therefore, the scarlatinal toxins became so modified by formaldehyde that a 1 in 10 dilution produced a skin reaction markedly less than the standard Dick test toxin (0·2 c.c. of 1 in 2000 dilution), these antigens were used to immunize Dick-positive reactors.

Toxin modified by the addition of 2% formalin first became available. Preliminary inoculation experiments with graduated doses of this toxoid were made on 4 individuals with markedly positive Dick tests, and it appeared that 1 c.c. of the undiluted toxoid could be tolerated without general reaction. Accordingly 16 Dick-positive reactors varying in age from 4 to 12 years were chosen and given 3 inoculations of 1 c.c. of undiluted toxoid at weekly intervals. The results obtained when the Dick test was reapplied 1 month after immunization showed that 9 out of 16 individuals had become completely Dick-negative, and that the degree of reaction produced by the Dick test was considerably reduced in other 5. By chance 2 cases who gave persistent markedly positive reactions subsequently developed scarlet fever. The first case, a female, aged 5 years, became ill 20 days after the final Dick test, and with the development of the disease the area of skin which had previously been

the site of the Dick test reaction became again markedly inflamed; the second case developed scarlet fever 6 weeks after the final Dick test but the positive phase of the test did not reappear.

When the toxin containing 1% formalin became sufficiently modified preliminary inoculation tests on susceptible individuals showed that 1 c.c. of undiluted toxoid could be tolerated. At a school in the City 150 children aged 7 years and over were Dick-tested, and 79 found to be positive reactors.

Accordingly these 79 individuals, together with 19 under 7 years of age, were inoculated at weekly intervals with 0·5 c.c., 1 c.c. and 1·5 c.c. of undiluted 1% formalin toxoid. Two months later the parents were asked for permission to re-test, but unfortunately only permission to re-test 42 was obtained. The results of the re-tests showed the following:

Number over 7 years of age previously Dick-positive = 23
„ now negative reactors . . . . = 10
„ still showing positive reactions. . . . = 13

Although the number of those re-tested was small, the results definitely indicated that antitoxic immunity had been produced in 43% of the previously Dick-positive cases.

A final experiment was carried out with toxin modified by the addition of 0·5% formalin. Preliminary experimental inoculation with this material again showed that 1 c.c. of undiluted toxoid could be readily tolerated, the local reactions induced being of an exceedingly mild character.

At a school permission was obtained to immunize 40 children under the age of 7 years and to test, and, if necessary, immunize 134 children over 7 years of age. The Dick test showed that 74 gave a positive reaction and 60 a negative test. Accordingly 40 children under 7 years of age and 74 children over 7 years were immunized by giving 3 doses of 1 c.c. of undiluted toxoid at weekly intervals. Three months later the parents of the children who had been previously Dick positive were asked to give permission to allow a re-test to be made. The results obtained showed the following:

Total number re-tested (previously all positive) = 52
Number still giving a Dick-positive reaction . = 25
„ now negative to Dick test . . = 27

In addition a class of fourth-year medical students 44 in number submitted to the Dick test, and those who were Dick-positive received 3 inoculations at weekly intervals of 1 c.c. of the 0·5% formalin toxoid and a re-test was made 2 months later. The results were as follows:

Total number of students		44
Number giving positive Dick test . . .	32	
„ „ negative test	12	
Total number inoculated		32
Number re-tested and found positive . .	14	
„ „ „ negative . .	18	

Thus 52% of the children and 56% of the adults had been successfully immunized.

DISCUSSION.

The various experiments carried out on the modification of scarlatinal toxin have shown that many weeks are required to produce the anatoxin unless formalin is present in great excess. The action of formalin has been studied on two more samples of toxin and the results of the first experiment have been fully corroborated.

Immunization by means of toxin modified by 2% formalin is impracticable on a large scale, since the pain produced by the excess of formalin, although it only lasts for 5 to 10 minutes, is so severe as to preclude its use. When 1·5 c.c. of toxoid containing 1% formalin was used the children also complained considerably of pain. The discomfort caused on inoculating with the toxoid modified by 0·5% formalin was, however, trifling, and none of the children developed the scarlatinoid syndrome following an injection of any of the toxoids.

Although the percentage of individuals rendered immune to the Dick dose of toxin (0·2 c.c. of 1 in 2000 dilution of the same toxin for all tests and re-tests) has not been high, the results suggest that further experiments should be carried out. Further, it should be remembered that scarlatinal toxins cannot first be tested on animals, and those showing the best antigenic properties selected for immunizing human individuals.

SUMMARY.

Scarlatinal toxoid has been prepared by the addition of 2%, 1% and 0·5% formalin to scarlatinal toxin.

The immunizing properties of these various antigens have been studied.

BIBLIOGRAPHY.

Kinloch, J. P., Smith, and J., Taylor, J. S.—(1927) *J. Hyg.*, **26**, 327.
Sparrow, H., and Celarek, J.—(1927) *C.r. Soc. de Biol.*, **97**, 957.
Zoeller, C.—(1925) *Ibid.*, **92**, 244.

A METHOD OF STAINING BACTERIAL FLAGELLA.

JAMES CRAIGIE, M.B., CH.B.

From the Department of Bacteriology, University of St. Andrews.
(University College, Dundee.)

Received for publication February 1st, 1928.

THE method is essentially that of Zettnow with modifications.

In the first place, the organismal suspensions are fixed by formol before any attempt is made to stain them. This makes staining easier, and also appears to prevent shedding of the flagella, so that the relation of the flagella to the bodies of the bacteria is well demonstrated.

Secondly, special care is taken to eliminate electrolytes before mordanting.

Thirdly, the process of silver reduction is not completed, as the completion of this is liable to give dirty preparations, the flagella being unduly thick.

Fourthly, fading is guarded against and intensification is produced by exposure to gold solution, which is advised as the final procedure of the modified method.

In this, as in all methods of staining flagella, special care must be taken to prevent any grease being present on the slides. The slides should be handled only with forceps and it is advised that two pairs of forceps should be used, one reserved exclusively for handling slides with mordant so that no trace of mordant can find its way into the silver solution when applied to the slide.

The method used is as follows:

(*a*) Use 24-hour cultures on agar with plenty of water of condensation and pipette off the growth in the condensation water, which is at once dropped into "fixative solution." The fixative solution consists of saline containing 2% of formalin. Formolized phosphate buffer solution of pH_7 may be used instead of saline. Leave in fixative solution for 1 hour or, better, overnight, before attempting to stain. These formolized suspensions keep for months at room temperature.

(*b*) *To prepare glass-ware.*—Slides or tubes used should be of hard glass, and are cleaned by immersion in sulphuric acid bichromate mixture without heat, being stored in this until required. Wash under tap, dry with grease-free fabric, roast in Bunsen flame, and allow to cool.

(*c*) *Preparation of films.*—In a clean tube put distilled water and add thereto the formolized suspension until faint opacity appears. Spread gently a small loopful of this on a slide cleaned as above. Dry at 37° C., the slide being inclined so that varying degrees of thickness of suspension are obtained. Then heat at 90–100° C. for 5 minutes or longer. (This can be done conveniently on the lid of a small steam sterilizer such as is used for sterilizing pipettes or syringes.)

(*d*) *Removal of electrolyte.*—Place in jar of distilled water for 5 minutes and wash with distilled water. Dry and heat again at 90–100° C., when preparations may be mordanted.

(*e*) *Mordanting.*—(i) Preparation of mordant: Zettnow's mordant is used. Dissolve 10 gm. of tannic acid (this must be "light and pure," *not* of commercial quality) in 200 c.c. of distilled water, heat to about 60° C., and slowly add, with frequent agitation, 30 c.c. of 5 % aqueous solution of tartar emetic. A crystal of thymol is necessary if the mordant is to be stored.

(ii) Application of mordant: The slide is flooded with mordant and heated for 5 to 10 minutes at 90–100° C. When doing this the slides should be under constant observation, and when any tendency to drying of the mordant is observed at the edges of the slides more mordant should be run on and guided along the dry edge with a small glass rod. Remove slide and flood off mordant under tap. Any dry mordant at the edges of the slides—this is easily seen, as it becomes white on cooling—must be removed with a clean cloth. Wash in distilled water.

(*f*) *Silvering.*—(i) Dissolve 1 gm. silver sulphate (B.D.H. brand used) in 250 c.c. of distilled water and store in light-tight bottle.

(ii) To 20 c.c. of this silver sulphate solution add mono-ethylamine solution (33 % B.D.H.) until the resulting opacity *just* clears up. More silver may be added if the ethylamine has been added in excess. About 4 to 5 drops of ethylamine are required for 20 c.c. of silver solution. Flood slides with "silver ethylamine" mixture thus prepared, and warm over tip of luminous flame till preparation just begins to steam. Allow to continue steaming, *but do not overheat,* until the preparation turns brown or black according to degree of reduction. It is emphasized that to obtain the best preparations heating should not be continued after the preparation has turned brown. If a black metallic cloud appears in the solution, wash off and replace with fresh "silver ethylamine." When sufficiently reduced flood off under tap, care being taken so to flood the slide that no metallic film settles on the preparation. Wash in distilled water. (If desired one can mount the preparation after drying, but such preparations fade rapidly, especially if the silver has not been completely reduced, and it has already been noted that complete reduction does not give optimum preparations.)

(*g*) *Gold "toning."*—Prepare "toning" solution by adding 20 drops of 1 % solution of gold chloride to 20 c.c. of distilled water. The gold solution was made from ordinary photographic gold chloride. Immerse slides in this weak solution of gold chloride and expose to daylight for 30 minutes or longer. Wash and dry.

[When greater density and contrast is desired the following alternative to gold "toning" (*g*) may be adopted with advantage.

Immerse slide for 5 minutes or longer in a 0·1 % solution of uranium chloride. Wash and apply the following developer for 1 to 2 minutes:

Pyrogallic acid (Merck), 0·5 % aqueous solution, 10 c.c.
Liquor ammoniæ fort., 3–5 drops.
Mix and use immediately.

Wash slide and dry.

This developer may be applied also to the gold preparations].

(*h*) *Mounting preparations.*—Preparations are mounted in balsam without solvent, the balsam being rendered fluid by heat. This method gives very delicate permanent preparations.

PANCREATIC FAT NECROSIS: A CHEMICAL STUDY.

FREDA K. HERBERT.

From the Biochemical Department, Hospital for Sick Children, Great Ormond Street, and the Department of Chemical Pathology, St. Bartholomew's Hospital.

Received for publication February 15th, 1928.

THE work here described was undertaken in order to compare the results of analysis of the fat in cases of pancreatic fat necrosis with those obtained in sclerema neonatorum. Sclerema neonatorum is characterized by lesions in the subcutaneous tissue which in some respects resemble fat necrosis; in these lesions neutral fat crystals are deposited, and there is no appreciable hydrolysis of the fat (Harrison, 1926; Kohnstam and Herbert, 1927). It was therefore decided to make chemical studies to test the long-accepted view that hydrolysis of the fat occurs in pancreatic fat necrosis—a belief which has hitherto been based only on histological and histochemical evidence. This evidence is: Histochemical work by Langerhans (1890); Benda's reaction (Benda, 1900); and the results of staining by Nile blue sulphate (Lecene and Moulonguet, 1925). The part played by the pancreatic ferments has been established experimentally by Wells (1903); his results point very strongly to a combined action of trypsin and lipase, but he could not make the evidence for this quite complete, as he was unable to obtain pancreatic lipase free from trypsin, and lipase from other sources (liver and serum) failed to produce fat necrosis when injected alone or with pancreatic trypsin.

PRELIMINARY HISTOLOGICAL AND HISTOCHEMICAL WORK ON OMENTAL FAT OF HUMAN ACUTE PANCREATITIS WITH FAT NECROSIS (FOUR CASES).

1. *Crystals in the Necrotic Areas.*

Determinations of the melting-point of crystals seen in unstained frozen sections, and of the solubility of these crystals in various solvents, were made, using the polarizing microscope and a warm stage.

In all cases anisotropic, acicular crystals were present both in the necrotic and in the non-necrotic areas of the frozen sections. Those in the non-necrotic areas were not pathological, for such crystals are commonly seen in fatty tissues, and consist of neutral fat. These "normal" crystals melted at 30–40° C., and, on cooling, reappeared at about 20° C. They were therefore not present as crystals during life. The crystals seen in the necrotic areas had a higher melting-point than those in the non-necrotic fat. It was found that these crystals were not all of the same nature, and this made it impossible to obtain much information from solubility experiments. Some were found, melting at

45°–50° C., and soluble in the fat solvents. Others remained unmelted at 60° C. and were insoluble in alkaline alcohol, even when heated, and therefore could not have been neutral fats or fatty acids.

2. *The Presence of Soap in the Necrotic Areas.*

An amorphous substance was present, which had a milky appearance under the crossed Nicol's prisms. It was insoluble in dilute aqueous hydrochloric or acetic acid.

On treatment with *acid alcohol* (1% HCl in 95% alcohol) this material dissolved at once in the cold.

On treatment with *acid alcohol* (1% HCl in 60% alcohol) the immediate result was the appearance of numbers of short, fine, anisotropic acicular crystals, which dissolved when the section was warmed with the acid alcohol, and reappeared on cooling. If the section was then washed with water, to remove the alcohol, the crystals could be studied further. Their melting-point was 45°–50° C., and they were soluble in ether.

Alcohol (50%), acidulated with acetic acid, had the same effect in producing these crystals from the amorphous material.

These results suggested very strongly that the amorphous masses consisted of soap. On treatment with acid alcohol fatty acids were liberated, and dissolved at once in the cold if concentrated alcohol was used, but only on heating when the alcoholic solution was more dilute.

Since fatty acid crystals are so readily formed when soaps are treated with acid and dilute alcohol, it is essential that all the reagents used in the preparation and staining of frozen sections (alcohol, formol, and the gum solution used in freezing) should be neutral. They are best neutralized with soda, using phenolphthalein as indicator.

3. *Nile Blue Staining.*

Frozen sections stained by Nile blue sulphate showed masses of deep-blue-stained material in the necrotic areas.

4. *Benda's Reaction.*

Technique.—The material was fixed in 10% neutral formol, and was then treated with Weigert's neuroglia mordant for 24 hours at room temperature. Frozen sections were cut, and counterstained with Sudan and hæmatoxylin, or as desired. (Weigert's neuroglia mordant contains 5% neutral copper acetate, 5% acetic acid, and 2½% chrome alum; it is made up by adding the alum to the water, heating to boiling-point and adding the acetic acid and the powdered acetate.)

Berner (1907) has devised a method whereby material stained by Benda's method can be cut in paraffin without destroying the green crystals. The material is fixed in formol and treated with Weigert's neuroglia mordant as above. It is dehydrated with 70% and 90% alcohol, and passed through aniline oil, acetone and benzene. It is then embedded in paraffin, and sections are cut. The paraffin is removed with benzene, acetone and 70% alcohol,

and the sections are counterstained with hæmatoxylin. They must not be mounted in Canada balsam, but any of the media in use for mounting frozen sections is suitable.

In such paraffin sections the green crystals were seen in the necrotic areas, and the histology could be better studied than in frozen sections.

Benda's reaction was positive in all cases. The necrotic areas appeared to the naked eye as conspicuous green masses, and in the sections the tufts of green, acicular, anisotropic crystals were seen. The solubility of these crystals was tested both in the frozen and paraffin sections, and was as follows: Soluble in ether and benzene, sparingly soluble in alcohol, and insoluble in light petroleum.

Copper stearate and palmitate were soluble in benzene and ether, sparingly soluble in alcohol, and insoluble in light petroleum. Copper oleate was soluble in light petroleum as well as in benzene, ether and alcohol. The solubility of the green crystals in the sections is therefore consistent with the view that they consist of copper stearate and palmitate, formed from free fatty acid or soap present in the necrotic areas.

5. Histology of Fat Necrosis as Seen in the Paraffin Sections.

The process had a lobular distribution. The cells in the affected areas, especially at the periphery of the lobules, contained an amorphous substance, which sometimes stained with eosin and sometimes with hæmatoxylin, and which either completely filled the cell, or formed a crescentic or annular mass in the periphery of the cell. In the same areas (in sections treated by Berner's method) the green copper crystals were found.

There was usually some inflammatory reaction, but it was not marked; the cells in the exudate were lymphocytes and mononuclear cells.

CHEMICAL INVESTIGATIONS IN EXPERIMENTAL FAT NECROSIS.

Experimental fat necrosis was produced in rats and cats by intraperitoneal injections of commercial pancreatin. The usual dose was 5 c.c. of a 5% suspension for rats, and 10 c.c. of a 10% suspension for cats. These injections never failed to produce fat necrosis, but the extent of the necrosis varied considerably in the different animals. In an extensive case the characteristic distribution was as follows: Masses of fat necrosis were abundant in the omentum and mesenteries, and in the retroperitoneal fat along the outer borders of the psoas. From the lower end of the affected retroperitoneal fat the condition spread into the fat attached to the epididymis and into the subcutaneous fat of the abdominal wall, where small masses were seen along the line of the vessels. Above, where the affected retroperitoneal tissue met the diaphragm, fat necrosis was present, and the masses were abundant along the line of attachment of the diaphragm to the costal margin. They were also found along the lines of the ribs on the external surface of the intercostal muscles and over the xiphisternum. Occasionally a few necrotic masses were found within the thorax—in the pericardial and mediastinal fat.

There was usually a blood-stained peritoneal exudate, but seldom an actual peritonitis.

This extensive fat necrosis was easily produced in rats. In general in cats the lesions were not so widespread, but cats were used for all the chemical work, because rats did not provide enough material. The animals were killed 20–24 hours after the injection. Omental fat from two human cases of acute pancreatitis was also analysed.

Chemical Methods.

The omental and mesenteric fat was taken, and divided into two portions. The first (A) was cut up into small pieces, spread on a clock glass, and dried to constant weight in a vacuum desiccator. The second (B) was cut up and dried by grinding with sand and anhydrous sodium sulphate, to form a powdery mixture. Both samples were extracted with ether for 3–4 days in a Soxhlet apparatus. The ether-soluble portion after evaporation of the ether (the "ether-extract") was stored in a vacuum desiccator over $CaCl_2$ and paraffin shavings until constant weight was reached. The ether-insoluble portion ("ether-extracted residue") from (A) was similarly stored.

Drying without sodium sulphate is very slow, and marked hydrolysis may occur while the tissue is in the desiccator. The ether extract from fat so treated is therefore unsuitable for determination of the acid value. But since an ether-extracted residue unmixed with sodium sulphate was required for determinations of calcium and of soaps, the fat had to be divided into two samples as described. The ether extract from (B) was used for the determinations of the acid value, saponification value, and iodine value; the ether-extracted residue from (A) was used for determinations of calcium and of soaps.

Ether extract.

The acid value and saponification value were determined by the usual standard methods, and the iodine value by Wij's method.

Ether-extracted residue.

Total calcium.—The ether-extracted residue was ashed, the ash taken up in aqueous hydrochloric acid and the calcium determined by Kramer and Tisdall's method.

Calcium as soap.—The soaps of Ca, K, and Na are soluble in amyl alcohol (Wells, 1906).

For determination of the Ca present as soap, the ether-extracted residue was extracted with amyl alcohol in a Soxhlet apparatus heated on a sand-bath (8–9 hours). The amyl alcohol solution was evaporated to small volume, and transferred quantitatively to a crucible. The remainder of the amyl alcohol was driven off by heating on a sand-bath, and the residue ashed in a furnace. The Ca in the ash was estimated by Kramer and Tisdall's method.

Other soaps.—In most cases no attempt was made to estimate the total soap present. In one case this was done as follows: The ether-extracted residue was extracted with amyl alcohol. The solution in amyl alcohol was taken to dryness by distillation under reduced pressure. The residue was digested with hot aqueous hydrochloric acid, and the mixture extracted with light petroleum. The petroleum solution was washed with water until it was

free from hydrochloric acid, and then titrated with alcoholic potash. In this way the total fatty acid, which had been present in the tissue as soap, was estimated.

RESULTS.

Saponification values.—In six normal cats the figures ranged from 196 to 204 mgm. KOH per 1 gm. ether extract; in four cats with varying degrees of fat necrosis, 192–200; in one sample of normal human omentum, 198; and in one human case of fat necrosis (acute pancreatitis), 196·5. There was therefore no significant difference between the normal and fat necrosis results.

Iodine values.—These were: In four normal cats, 56–63 gm. iodine per 100 gm. ether extract; in five cats with fat necrosis, 57·9–62·1; in one sample of normal human omentum, 68; and in one case of human fat necrosis, 65. There was, therefore, no change in the iodine value. Nevertheless it is possible that there was a diminution of unsaturated fatty acid compounds in the necrotic areas, since these areas do not stain with osmic acid (Adami, 1909). Such a change would have to be very extensive in order to affect the iodine value of the whole sample, which included a great deal of unaffected fat. The iodine value of the normal fats varied appreciably, and it would scarcely be expected that the disappearance of unsaturated fatty acid compounds from the necrotic areas would bring the iodine values of the samples below the normal limits.

Acid values.—The acid value in eight normal cats' omental fat varied between 3 and 4·8 mgm. KOH per 1 gm. ether extract. The one normal human case gave a figure of 1·5.

In the six cats in which the degree of fat necrosis was slight or moderate the acid values ranged between 3 and 13·7. Of five cats in which the necrosis was extensive, in one the acid value was 11, in the rest the figures ranged between 20·6 and 29·5. In the two human cases of fat necrosis the values were 13·0 and 36·7.

The degree of hydrolysis was therefore considerable in the extensive cases, as is seen by comparing the acid values with the saponification values, both of which are expressed as mgm. KOH per 1 gm. ether extract. The normal average saponification value was approximately 200—that is to say, complete hydrolysis of the triglycerides would correspond to an acid value of 200. An acid value of the order of 20–30, therefore, indicates hydrolysis of 10–15% of the fat, with liberation of free fatty acid.

Calcium figures.—The total calcium, as Ca per cent. of the ether-extracted residue, was as follows: In eight normal cats, 0·11% to 0·28%; in one sample of normal human omentum, 0·14%; in six cats with fat necrosis of "slight" or "moderate" extent, 0·16–0·40%; in four cats with "extensive" fat necrosis, 0·50–1·05%; and in one human case with extensive fat necrosis, 1·45%. There is thus a marked rise in the total calcium in the "extensive" cases, but the calcium figures did not run exactly parallel with the acid values.

Soaps.—The increase in the total calcium was not due mainly to the formation of calcium soap. In some cases no calcium, soluble in amyl alcohol, was detected; in the cases in which it was found the quantity was very small, ranging, in 6 cases, between 0·053 and 0·089% of the ether-extracted residue.

These figures did not run parallel with the figures for the total calcium, or with the figures for the acid values.

The efficiency of the extraction by amyl alcohol was tested on calcium palmitate, which was completely taken up when extracted under the same conditions. If it may be assumed that the soap in the tissues has a similar solubility, the amyl alcohol extract will contain all the calcium which was present in the tissue as soap. On the other hand, the amyl alcohol extract may contain some other calcium salts in traces, since the phosphate and carbonate are not absolutely insoluble in amyl alcohol. The figures may therefore be too high, but it is clear that the calcium present as soap is not greater than that indicated by the figures.

The total soap was estimated in one cat. It was found that the amount was very small in proportion to the free fatty acid. Moreover the calcium soap was only a small part of the total soap. The acid value of the ether extract was in this case 23·7, and the calcium soluble in amyl alcohol was ·06 % (approx.) of the ether-extracted residue. In order to compare the free fatty acid with the soap, the figures have been re-calculated as c.c. normal acid per 100 gm. of dried omental tissue. The figures were:

Free fatty acid:	37·79 c.c.	normal acid	per	100 gm.	dry omental tissue.
Total soap:	3·36 ,,	,,	,,	,,	
Calcium soap:	0·54 ,, (approx.)	,,	,,	,,	

(It is here assumed that all the calcium soluble in amyl alcohol is calcium soap.)

The fact that the amount of soap formed is so small in proportion to the amount of free fatty acid is interesting, because Klotz (1905, 1906) has put forward the view that the formation of soap is the first step in the deposition of calcium in many calcifying lesions, including atheroma of the aorta. Baldauf (1906, 1907) and Wells (1906, 1907, 1910, 1911) have brought forward strong evidence against this view as a general theory of calcification, but Wells (1911) has admitted that calcium soaps may be formed in pancreatic fat necrosis and in calcifying lipomata.

The present work shows that even in pancreatic fat necrosis the amount of calcium soap is very small, although there is a marked rise in the calcium content of the tissue.

SUMMARY.

Histochemical observations are described, which confirm the accepted opinion that soap is formed in the necrotic areas in pancreatic fat necrosis. Possibly neutral fat, fatty acid and soaps are all present in crystalline form. There is certainly more than one type of crystal, but histochemical identification is unsatisfactory.

By chemical analysis it has been shown that hydrolysis and saponification of the fat do occur in pancreatic fat necrosis, but the hydrolysis is mainly an acid hydrolysis, and very little soap is formed. There is a marked rise in the calcium content of the omental tissue when the fat necrosis is extensive, but only a minute portion of this calcium exists as soap.

For human material I am indebted to Prof. M. J. Stewart, Dr. J. W. McNee, Mr. B. W. Williams, Dr. C. F. T. East, Dr. L. F. Hewitt and Sir Bernard Spilsbury.

I wish especially to express my thanks to Dr. G. A. Harrison. The work was undertaken at his suggestion and carried out in his laboratories, and I am much indebted to him for advice and criticism. Dr. Harrison himself made all the animal injections.

REFERENCES.

ADAMI, J. G.—(1909) "Principles of Pathology," 'General Pathology,' p. 903. London.
BALDAUF, L. K.—(1906) *J. Med. Res.*, **15**, 355.—(1907) *J. Amer. Med. Assoc.*, **49**, 642.
BENDA, C.—(1900) *Virch. Arch.*, **161**, 194.
BERNER, O.—(1907) *Ibid.*, **187**, 360.
HARRISON, G. A.—(1926) *Arch. Dis. Child.*, **1**, 63, 123.
KLOTZ, O.—(1905) *J. Exp. Med.*, **7**, 633.—(1906) *Ibid.*, **8**, 322.
KOHNSTAM, G. L. S., AND HERBERT, F. K.—(1927) *Arch. Dis. Child.*, **2**, 349.
LANGERHANS, R.—(1890) *Virch. Arch.*, **122**, 252.
LECENE, P., AND MOULONGUET, P.—(1925) *Ann. d'anat. path.*, **2**, 193.
WELLS, H. G.—(1903) *J. Med. Res.*, **9**, 70.—(1906) *Ibid.*, **14**, 491.—(1907) *Ibid.*, **17**, 15.—(1910) *Ibid.*, **22**, 501.—(1911) *Arch. Int. Med.*, **7**, 721.

THE RELATION BETWEEN FATNESS AND STERILITY.

A. S. PARKES,

Beit Memorial Research Fellow,

AND

J. C. DRUMMOND.

From the Department of Physiology and Biochemistry, University College, London.

Received for publication February 8th, 1928.

IT has long been known that a certain relation exists between a condition of extreme fatness in mammals and complete or partial sterility. The exact nature of this relationship is difficult to arrive at. On the one hand, there are facts which suggest that fatness is an exciting cause of sterility, while, on the other hand, other facts suggest that the sterility is primary, and that the fatness supervenes as a result of the metabolic disturbances produced by the elimination of the reproductive functions.

In the domestic animals intensive fattening may lead to aberration or entire cessation of the œstrous cycle and to degenerative ovarian changes (Marshall, 1922). Analogous data may be drawn from anthropological sources. In certain primitive races, notably in Melanesia and in Calabar, certain fattening

customs are found which have interesting biological effects. In Old Calabar (Malcolm, 1925) all young girls of any social standing are subject to two or three years' fattening treatment, during which time they are fed on the most oily foods and prohibited from taking any form of exercise. The result is that enormous amounts of abdominal and subcutaneous fat are laid down, and many of these females subsequently prove to be completely sterile. In some cases the issue is complicated by the coincident practice of mutilations, such as clitoridectomy, but there seems no doubt that extreme fatness in some primitive races is associated with a marked degree of sterility.

The evidence that sterility may be followed by the extreme deposition of fat is also considerable. In mammals the operation of gonadectomy is usually followed by the abnormal laying down of flesh and fat—a fact which is taken advantage of agriculturally in the production of fat beasts. The operation of gonadectomy fails to distinguish, of course, between the elimination of the internal secretory function of the gonad and of the gametogenetic function, but since X-ray sterilization (Parkes, 1927) also very often leads to the same result, especially in the female, it may be supposed that the effect of gonadectomy is not solely due to the elimination of the internal secretions. In laboratory rodents, particularly mice, the period during which the female will breed is strictly limited. Under laboratory conditions female mice tend to become loaded with mesenteric fat after the first few litters, and either as a cause or as a result of this, aberrations of the œstrous cycle occur and the animals become practically sterile. In animals which are not allowed to begin breeding at the normal age of puberty this process begins much sooner, as also in young females which for any reason fail to breed soon after puberty.

These diverse facts suggest most strongly that fatness may be both a result and a cause of sterility, and it was thought that further light might be thrown on the problem by examination of the fertility of animals kept on artificial diets containing a large proportion of fat. The experiments described in this paper were performed on rats fed on such diets, and tend to show that appreciable degrees of obesity are not necessarily incompatible with the normal functioning of the reproductive organs. The results, therefore, support the supposition that fatness may be a result rather than a cause of sterility.

Methods and Materials.

Scheme of experiments.—Eighty-two female rats in all were used. All these were kept on natural food for some weeks, during which time the œstrous cycles were observed for control data. Sixty-three animals were then put on to the fat diets described below, the rest being kept on normal food for mating with dieted males. Of the dieted females a number failed to thrive and died, while a few more obviously failed to respond to the treatment and were killed. After 95 to 116 days on diet 50 rats were available for further experiment: 20 of this number were mated with normal males in order that the effect of the diet on fecundity, fertility and sex-ratio could be observed; the remaining 30 animals were killed in order to determine the degree of fatness attained.

Twelve male rats were put on to the fat diets. One died early and one was

dissected after 29 days, in order to ascertain what effect the diet was having. The remaining 10 were mated with normal fertile females.

Diet.—As it was deemed inadvisable to put the animals suddenly on to a diet with a very high content of fat, a graduated series of diet was used. The first 3 contained an increasing percentage of hardened fat, and the last 2 had a small percentage of wheat embryo oil added in order to provide the reproductive vitamin E. The composition of these diets was as follows:

	I.	II.	III.	IV.	V.
Casein	20	20	20	20	20
Starch	53	48	43	42	41
Salt mixture	5	5	5	5	5
Marmite	5	5	5	5	5
Hardened fat	15	20	25	25	25
Cod-liver oil	2	2	2	2	2
Wheat embryo oil	0	0	0	1	2
	100	100	100	100	100

Three days on each of the first four diets was allowed before passing on to the next.

Criterion of degree of fatness.—Chemical estimation of the percentage of fat in the carcass was considered too laborious, and it seemed preferable to arrive at some simpler system. Since a large amount of the body fat in rats is mesenteric and perinephritic, it was decided to dissect the abdominal fat from each carcass as completely as possible, weigh it, and calculate the amount as percentage of body-weight (to one decimal place). The abdominal fat percentage (A.F.P.) was then used as the index of fatness attained.

Detection of œstrus, etc.—The detection of all phases of the œstrous cycle, including copulation and pregnancy, was carried out by means of the vaginal smear technique described for the rat by Long and Evans (1922).

Degree of Fatness Attained.

Very early in the experiments it was clear that no abnormally heavy rats would be produced. A gain in weight of 10–15 gm. was found in many animals early in the experiment, but nothing abnormal was subsequently found in the weight curves, the weights remaining fairly steady at 180–220 gm.

Control figures for the abdominal fat percentage were obtained from 58 normal rats which had been left on a complete artificial diet. The figures varied from 0·7 to 5·4, the mean being 2·3. Data are available for 47 dieted rats, and the fat percentage in this series varied from 2·0 to 8·8, the mean being 4·8.

The distributions of the percentages give the values shown in Table I:

TABLE I.

	Control rats.	Fat rats.
Mean	2·3	4·8
Standard deviation	·91	1·51
Probable error	± ·08	± 0·15

The difference in A.F.P. is, therefore, 2·5 ± 0·18. The difference is 13·9 times its probable error and strongly significant.

From these figures it is evident that the effect of the dieting was to produce an increase in fatness represented by a highly significant increase of more than 100% in A.F.P., and thus to achieve the object in view.

The Effect of Fatness on the Œstrous Cycles.

In the 50 animals which were subsequently put on to the fat diets, 279 œstrous cycles were first observed. The length varied from 3 to 23 days, but the abnormal ones were very rare. The mean length of these pre-treatment cycles was 5·71. The cycles occurring during the time when the animals were being acclimatized on the graduated dietary are not included as occurring during treatment. While the rats were on the full fat diet, 874 cycles were observed, varying in length from 3 to 17 days, and having a mean of 5·24.

The distributions for the length of cycle give the values shown in Table II:

TABLE II.

	Cycles on natural diet.	Cycles on fat diet.
Mean	5·71	5·24
Standard deviation	2·40	1·77
Probable error	±0·09	±0·04

The difference in the length of the cycle during treatment is thus 0·47 ± 0·11 days. Although this difference represents 4·3 times its probable error and is thus of some statistical significance, it can have but little biological meaning. Certainly the periodicity of œstrus cannot, from these data, be said to have been deranged. It must be supposed, therefore, that the œstrous cycle remains unchanged in animals fattened up to an extent represented by more than 100% increase in abdominal fat. That this conclusion, drawn from the vaginal cycle, also holds good for the ovarian cycle is abundantly shown below.

Breeding Performance of Fat Animals.

The 20 females mated after a considerable period on the fat diet, at the time when the remaining animals were killed for estimation of the A.F.P., proved on the whole to be quite fertile. The males with which they were mated had hitherto been on natural diet, but while with the females they had, of course, to be kept on the fat diets. Table III shows the individual histories of the mated fat females:

TABLE III.—*Breeding Performance of Fat Females.*

No. of rat.	Time on diet at copulation.	Size of litter.	Sex of litter. ♂	Sex of litter. ♀
F 4	118 days	4	1	3
F 8	128 ,,	1	Dissected during pregnancy	
F 11	Mated at 112 days but no copulation observed	...	...	...
F 12	114 days	2	1	1
F 15	Mated at 112 days but no copulation observed	...	...	...
F 20	Ditto	...	...	...
F 22	133 days	Copulation infertile	...	...
F 24	117 ,,	5	2	3
F 26	117 ,,	7	2	5
F 29	115 ,,	4	3	1
F 31	134 ,,	7	Dissected during pregnancy	
F 33	139 ,,	6	Ditto	
F 38	127 ,,	7	,,	
F 41	118 ,,	7	3	4
F 43	Mated at 95 days but no copulation observed	...	...	...
F 46	112 days	6	3	3
F 49	Mated at 95 days but no copulation observed	...	...	...
F 52	102 days	8	6	2
F 59	103 ,,	7	4	3
F 63	Mated at 95 days but no copulation observed	...	...	...
		71	25	25

Of these 20 females, therefore, 14 copulated and 13 produced litters, totalling 71 young—an average litter size of 5·5. The sex-ratio of those young which were sexed showed no aberration. The A.F.P. of the 7 fat females which failed to breed showed no detectable peculiarity, so that they cannot be supposed to have been particularly fat specimens. These breeding data make it quite clear that no suggestion of uniform sterility resulted from the 100% increase in fatness.

The breeding histories of the fat males with normal females are, however, much more suggestive of abnormality, as shown in the following table:

TABLE IV.—*Breeding Performance of Fat Males.*

No. of rat.	Time on diet at copulation.	Size of litter.	Sexes. ♂	Sexes. ♀
F♂ 1	102 days	2	1	1
F♂ 2	Mated at 92 days but no copulation or pregnancy observed	...	...	...
F♂ 3	Mated at 92 days but died soon after	...	...	...
F♂ 4	Mated at 92 days but copulation infertile	...	...	...
F♂ 5	59 days	8	5	3
F♂ 6	Died before mating	...	...	...
F♂ 7	Mated at 92 days but no copulation or pregnancy observed	...	...	...
F♂ 8	Died before mating	...	...	...
F♂ 9	Mated at 92 days but no copulation or pregnancy observed	...	...	...
F♂10	99 days	3	1	2
F♂11	Mated at 92 days but no copulation or pregnancy observed	...	...	...
F♂12	Mated at 103 days but no copulation or pregnancy observed	...	...	...

From this table it will be seen that only 4 out of 10 of these animals copulated with the normal females, but 3 out of the 4 copulations resulted in litters. This disinclination to copulate may be put down to the lethargy shown by the fat animals. Since the male is the active sex in coitus, this lethargy might well inhibit copulation between fat males and normal females without necessarily having the same effect on the reciprocal cross. This interpretation of the partial sterility of fat males is supported by the fact that spermatozoa capable of being made motile were found in each epididymis of all the fat males.

SUMMARY.

1. To endeavour to elucidate the relation which has often been noted between fatness and sterility, rats were kept on artificial diets containing a large percentage of fat, in the hope of producing large amounts of body-fat.

2. The animals kept on fat diets increased their abdominal fat percentage to over 100 % more than was found in normal rats kept on natural foods.

3. This increased fatness, however, failed to produce any obvious effect on the œstrous cycle. The mean length of the 279 œstrous cycles observed before going on to the fat diets ($5{\cdot}71 \pm 0{\cdot}09$ days) was hardly distinguishable from

that of the 874 cycles occurring during the period of fat feeding (5·24 ± 0·04 days).

4. Similarly, after about 4 months on the fat diets the fertility of the females was hardly affected, 65 % of the animals showing a normal breeding performance.

5. In the male, however, more effect of the fat diet was found, a majority of the animals failing to breed with normal females. Since the testes, however, appeared normal, this was probably due to lethargy caused by their fat condition rather than to any direct influence on the gonads.

6. From these results it is inferred that an excessive degree of fatness does not necessarily lead to sterility, and that fatness is more often a result than a cause of sterility.

The expenses of the investigation were defrayed from grants from the Medical Research Council, to whom our thanks are due.

REFERENCES.

LONG, J. A., AND EVANS H. M.—(1922) *Memoirs of the University of California*, No. 6.
MALCOLM, L. W. G.—(1925) *Man*, **25**, 113.
MARSHALL, F. H. A.—(1922) 'The Physiology of Reproduction.' London (Longmans).
PARKES, A. S.—(1927) *Proc. R. Soc.*, B, **101**, 71.

THE INCIDENCE OF *B. TETANI* IN HUMAN FÆCES.

JOHN C. KERRIN, M.B., CH.B., D.P.H.,

Carnegie Teaching Fellow.

From the Department of Bacteriology, Marischal College, Aberdeen.

Received for publication March 12th, 1928.

THE occurrence of tetanus spores in human fæces has been reported by several workers, but the results vary greatly in regard to the incidence of positive results.

Ten Broeck and Bauer (1922) in Peking found that the stools of Chinese patients were positive for tetanus bacilli in 34·7 % of cases, whereas Kahn (1924), in an examination of the fæces of 72 persons in New York, failed to find tetanus.

Fildes (1925), in this country, examined 200 stools and found that the number of carriers of tetanus spores was 1 %. His method was to incubate

about 2 gm. of fæces into boiled blood broth and incubate aerobically for 2–4 days. Two drops of the culture were added to the condensation water of a fairly dry peptic blood-agar slope (1920), which was then incubated anaerobically. If *B. tetani* were present, it climbed higher up the surface of the agar than the other organisms as a delicate filamentous growth and could be picked off from the top in pure culture.

Bauer and Meyer (1926), in California, using a different method, found that the percentage of human carriers of tetanus spores was 24·6. They comment upon the fact that while Fildes was only able to find tetanus in 1 % of fæces by his method, Tulloch (1919–20) found it in 16 % of the civilian population, and they themselves in 24·6 %, and suggest that these different results may be due to the methods employed. The method of Bauer and Meyer was to inoculate a bean-sized portion of fæces into beef heart medium, heat at 70–75° C. for 25 minutes, cool rapidly and incubate aerobically for 5–7 days. Smears were examined for the presence of tetanus-like organisms, and cultures which appeared to be positive were inoculated into Tulloch's exhaust medium (1919), to which a small fragment of fresh kidney had been added, and incubated for 5 days. Dilutions of this culture were made with saline, and deep tubes of liver agar were inoculated. Tetanus colonies could easily be recognized and fished. No culture which in film appeared to be positive failed to yield *B. tetani*. No atoxic strains were found.

In view of these conflicting results, it was decided to attempt to estimate the percentage of carriers of tetanus spores in Aberdeen and the surrounding districts. The methods employed were those of Fildes, and of Bauer and Meyer, with slight modifications. All specimens were inoculated into meat media and heated at 70–75° C. for 20 minutes. The tubes were cooled rapidly and incubated aerobically for 7 days. Films were then made, and any culture which contained bacilli with terminal spores in any way resembling *B. tetani* was inoculated into Tulloch's exhaust medium containing a piece of fresh kidney. This was incubated for one week under anaerobic conditions in a MacIntosh and Fildes anaerobic jar. The cultures from Tulloch's medium were (*a*) plated out on hormone agar (Bailey, 1925); (*b*) inoculated into the condensation water of a peptic digest blood-agar slope; (*c*) inoculated into a mouse.

(*a*) and (*b*) were incubated anaerobically. To ensure perfectly dry plates, the poured plates were dried in the incubator before inoculation, and the bottom of the anaerobic jar was covered with calcium chloride. As a control to these methods, a small portion of scar-tissue from a fatal case of tetanus was treated in the same way and pure cultures of *B. tetani* were easily obtained by both methods, although the primary culture in meat medium contained a variety of anaerobic organisms.

During this investigation 204 specimens of fæces were examined, a large percentage of which were from children in the Royal Hospital for Sick Children, and City Hospital, Aberdeen. Twenty-four specimens were from babies in Burnside Nursing Home. A considerable number of specimens showed the presence of organisms with terminal spores. In the primary meat cultures, organisms morphologically suggesting *B. tetani* were obtained in 36 cases. These were carefully investigated by the above methods, but in no

instance was *B. tetani* found either by repeated plating out or by the method of Fildes. Inoculation of mice with cultures in Tulloch's exhaust medium was performed with all suspicious samples, and negative results were uniformly obtained. These were controlled with the toxic strain of tetanus obtained from the human case. *B. putrificus* was met with fairly frequently and isolated in pure culture. It was especially common in the babies' stools.

DISCUSSION.

The results obtained in this investigation are in agreement with those of Fildes. The absence of *B. tetani* may be due to the fact that most of the specimens were from children, the majority of whom were town dwellers. It is quite probable that were the fæces of farm workers to be examined a different result would be obtained. The high percentage obtained by Ten Broeck and Bauer may be explained by the fact that the sanitary conditions in Peking are primitive, while in California, Bauer and Meyer were dealing with a rural population. The incidence of clinical tetanus in California is fairly high, in Aberdeenshire it is low. Most of the specimens examined in Aberdeen were pathological, a large number of them being from cases of Sonne dysentery.

SUMMARY.

204 stools have been examined for tetanus bacilli with negative results.

I have to express my thanks to the Medical Staff of the Royal Hospital for Sick Children, and to Dr. J. Smith, City Bacteriologist, Aberdeen, for providing me with the specimens of fæces.

REFERENCES.

Bailey, S. F.—(1925) *J. Infect. Dis.*, **36**, 341.
Bauer, J. H., and Meyer, K. F.—(1926) *Ibid.*, **38**, 296.
Fildes, P.—(1920) *Brit. J. Exper. Pathol.*, **1**, 129.—(1925) *Ibid.*, **6**, 62.
Kahn, M. C.—(1924) *J. Infect. Dis.*, **35**, 423.
Ten Broeck, C., and Bauer, J. H.—(1922) *J. Exper. Med.*, **36**, 261.
Tulloch, W. J.—(1919) *Proc. R. Soc.*, B, **40**, 529.—(1919–20) *J. Hyg.*, **18**, 103.

BLOOD PHOSPHORUS IN HEALTH AND DISEASE: III.—A. THE ALLEGED VALUE OF BLOOD PHOSPHORUS DETERMINATIONS IN SUSPECTED MALIGNANT DISEASE. —B. THE PHOSPHORUS DISTRIBUTION IN ANÆMIA, POLYCYTHÆMIA, AND LEUKÆMIA.

F. B. BYROM,

Eliza Ann Alston Scholar,

AND

H. D. KAY,

Beit Memorial Research Fellow.

From The Medical Unit, The London Hospital.

Received for publication February 10th, 1928.

A. THE ALLEGED VALUE OF BLOOD PHOSPHORUS DETERMINATIONS IN SUSPECTED MALIGNANT DISEASE.

A SATISFACTORY laboratory method for the diagnosis of malignant disease would have an importance that could hardly be exaggerated. A claim was made by Gröbly in 1921 that such a diagnosis could be made with a fair degree of probability by a determination of the total phosphorus content of the blood. He stated that, in patients suffering from cancer, a given volume of the whole blood contained, almost without exception, considerably more total phosphorus (Neumann's method) than that of a non-cancerous or of a normal individual. The difference was even more strikingly demonstrated when the total blood phosphorus was divided by the number of red cells. If the number of mgm. P_2O_5 in 10 c.c. of blood was divided by the number of erythrocytes in millions present in 1 c.mm., a quotient was obtained which varied in non-cancerous patients usually between 2·64 and 2·92 (average of 4, 2·75). In two cases of exophthalmic goitre it was as low as 1·42 and 1·44. In cancer cases this quotient was much higher, varying between 2·75 and 4·50 (average of 11, 3·58). He concluded that a patient with a phosphorus quotient of more than 3·17 was probably harbouring a malignant growth, and that, therefore, the determination of this quotient was of great value in diagnosis and treatment in cases of suspected cancer.

His findings were supported by Vorschütz and Vorschütz (1922), who determined Gröbly's quotient in a large number of cases of malignant disease and in a few control cases, and found a much higher quotient in the former. Patients with a higher quotient than 3·17, they agreed, were probably suffering from cancer. They advised, however, that the determinations be done on the red cells only, and stated that a figure of higher than 15 mgm. P_2O_5 per 10 c.c. washed red cells was indicative of cancer. Further support was given by Zerner (1924), who investigated 27 cases (4 not cancerous) by Gröbly's method.

All the cancerous patients except one had a Gröbly quotient of above 3, the average being 4·03.

If the data published by these three groups of workers are examined, the shortage of controls is most evident. Gröbly provides us with 6, as against 15 cases of cancer (4 post-operative); Vorschütz with 5 normal controls, 3 with icterus and 3 with other diseases, as against 9 cases of cancer; and Zerner with 4 only as against 23 cases of cancer.

Almost simultaneously with the publication of the last-mentioned paper, Buckman, Minot, Daland and Weld (1924), in a careful and well-controlled re-investigation of the question, came to the conclusion that the total phosphorus content of the whole blood, of plasma or of cells is not significantly altered in cancer, and that therefore the phosphorus quotient of Gröbly was of no value in the diagnosis of this disease.

We decided to subject these conflicting statements to a closer scrutiny.

1. *Theoretical.*

From the standpoint of our present knowledge of phosphorus compounds in blood, the derivation of a quotient by Gröbly's method is a dangerous procedure. Implicit in it is the assumption that all the blood phosphorus is in the red cells, which, although approximately true for the ester phosphorus, as has been repeatedly shown, is very far from being the case with the lipin, or with the inorganic phosphorus of the blood, both of which are present in the plasma in relatively large amounts. In 100 c.c. of whole blood, the absolute amount of inorganic or lipin P present in the plasma will depend entirely on the relative proportion of plasma to red cells. A quotient obtained, therefore, by dividing the total blood phosphorus by the number of red corpuscles in unit volume of blood involves a factor which increases with a diminishing red cell count, since the inorganic and lipin phosphorus of the plasma affect the quotient more and more seriously, raising it toward the "cancer level." On the basis of the Gröbly quotient, anæmic cases, or cases in which the lipin P of the blood is high, would tend to be diagnosed as cases of malignant disease. We have found, for example, that in several cases of obstructive jaundice of varying origin the blood lipin P rises from the normal figure of 12·0 mgm. P per 100 c.c. to as high as 20 mgm. or above. Such cases would undoubtedly, by Gröbly's criterion, be classified as cancerous. In view of the relatively high concentrations of P in the white cells, cases of chronic lymphatic and myeloid leukæmia would also find themselves in the same category (see section B). It is evident that, quite apart from cancer, the magnitude of Gröbly's quotient will depend, to a large extent, on the percentage volume and relative proportion of the various cells present in the blood.

2. *Experimental.*

It is of course possible that in spite of these theoretical objections, blood from patients suffering from cancer may, empirically, be found to be chemically distinguishable from normal, or from other pathological blood by its phosphorus content. We have, therefore, examined the blood of 28 cases of cancer and an adequate number of control cases, performing our phosphorus analyses as

described in the first paper of this series (Kay and Byrom, 1927). Blood-counts were made in the usual way, in duplicate, using a Thoma-Zeiss hæmocytometer, and counting 64 squares.

TABLE I.

Cases.		P in mgm. per 100 c.c. blood.							Red cells: millions per c.mm.	Gröbly quotient.	P index.
		Inorganic.	Total acid-soluble.	Ester.	Hydrolysable ester.	Non-hydrolysable ester.	Lipin.	Total.			
31 normal adults . .	Highest	3·8	30·6	27·5	9·4	19·3	13·8	42·9	6·1	1·98	59
	Lowest	2·4	22·9	19·6	5·0	12·2	10·4	34·6	4·4	1·44	48
	Mean	3·0	25·7	22·7	6·7	16·0	12·0	37·8	5·2	**1·67**	53
Various diseases, not exhibiting anæmia, non-cancerous (9)	Highest	3·5	30·0	27·4	7·8	20·5	16·0	43·5	5·50	2·04	67
	Lowest	2·5	25·0	21·4	5·9	15·1	11·1	32·5	4·70	1·40	48
	Mean	3·0	27·4	24·4	6·9	17·5	13·3	40·9	5·3	**1·81**	59
Cases exhibiting anæmia, but non-cancerous (14)	Highest	4·7	26·6	22·9	6·9	17·8	13·6	37·7	4·7	3·01	81
	Lowest	2·1	12·7	9·1	2·8	6·0	7·3	22·0	1·95	1·69	55
	Mean	3·2	19·7	16·5	4·8	11·7	11·0	31·7	3·1	**2·27**	65
Cancer cases without anæmia (18)	Highest	5·3	32·6	29·3	9·9	22·1	24·3	48·3	6·2	2·18	72
	Lowest	2·3	23·5	20·4	4·8	13·5	10·0	33·7	4·75	1·33	48
	Mean	3·2	27·3	24·1	7·0	17·1	12·2	39·8	5·33	**1·73**	57
Cancer cases with anæmia (10)	Highest	4·1	27·8	25·0	7·6	17·6	21·5	44·4	4·6	2·56	78
	Lowest	2·5	19·8	16·2	5·4	10·5	9·9	31·4	2·8	1·80	55
	Mean	3·2	22·7	19·5	6·2	13·3	13·1	35·5	3·85	**2·15**	66

Explanation of Headings in Tables I, IV and V.

Inorganic P = P present in form of salts of orthophosphoric acid.

Total acid-soluble P = P present in all phosphorus compounds which are not precipitated from the blood by coagulation of the proteins with trichloracetic acid.

Ester P = total acid-soluble P minus inorganic P.

Hydrolysable P = portion of ester P which is hydrolysed to inorganic P under certain conditions (Kay and Byrom, 1927).

Non-hydrolysable P = portion of ester P not so hydrolysed.

Lipin P = P of whole blood, or plasma, etc., which is soluble in alcohol-ether.

Total P = total P of whole blood as determined either by direct incineration or by adding lipin P to total acid-soluble P.

Organic P index = amount of ester P in mgm. per 100 c.c. red cells.

We have classified our results in five groups:

(*a*) Normal, healthy controls (31 cases).

(*b*) Non-anæmic controls suffering from diseases other than cancer (9 cases).

(*c*) Anæmic controls (14 cases).

(*d*) Cancer cases without anæmia (18 cases).

(*e*) Cancer cases with anæmia (10 cases).

Of these groups, details have already been given of (*a*) (Kay and Byrom, 1927). In Table I, highest, lowest and mean figures for the phosphorus partition, for Gröbly's quotient and for our phosphorus index are given for each group of cases.*

* Owing to the high cost of printing, complete analytical figures, comprising ten items for each of 69 cases, cannot be published here.

TABLE II.

		Total P in mgm. per 100 c.c. blood.	Red cell count: millions per c.mm.	Gröbly's quotient.
Gröbly's figures:				
Controls (4)	Highest	54·2	4·7	2·92
	Lowest	40·0	3·3	2·64
	Mean	45·9	3·8	**2·75**
Cancer cases (11)	Highest	108·9	6·5	4·53
	Lowest	45·6	2·5	2·74
	Mean	69·9	4·5	**3·58**
Zerner's figures:				
Controls (4)	Highest	65·4	5·0	2·9
	Lowest	35·4	3·4	2·1
	Mean	45·3	4·2	**2·45**
Cancer cases (23)	Highest	104·2	4·9	6·4
	Lowest	42·8	2·5	3·0
	Mean	61·8	3·5	**4·0**
Buckman et al. *figures:*				
Controls (28)	Highest	50·7	9·6	5·0
	Lowest	14·7	1·5	0·8
	Mean	31·6	4·3	**1·88***
Cancer cases (17)	Highest	47·6	6·0	2·55
	Lowest	8·6	1·8	1·05
	Mean	32 1	4·25	**1·71***
Present authors' collected figures:				
Controls,† normal + pathological (55)	Highest	55·2	8·7	3·01
	Lowest	22·0	1·95	1·40
	Mean	37·9	4·75	**1·85**
Cancer cases (28)	Highest	48·3	6·2	2 56
	Lowest	31·4	2·8	1·33
	Mean	38·3	4·80	**1·88**

* There is an arithmetical error in the figures for this quotient given by Buckman *et al.* in their 1924 paper, the values given being twice too high. In the above table the necessary corrections have been made.

† Includes all normals and all pathological controls.

The Gröbly quotient has been calculated strictly according to that author, *i.e.*:

$$\frac{\text{Mgm. } P_2O_5 \text{ in 10 c.c. whole blood.}}{\text{Number of red cells in millions per c.mm. blood.}}$$

The other phosphorus results are, however, shown as mgm. P per 100 c.c. blood.

Discussion.

It will be convenient at this point to collect together Gröbly's data and those of Zerner, and also the figures of Buckman, Minot, Daland and Weld for comparison. The total phosphorus figures have been recalculated in terms of P, and are shown in Table II together with our own collected figures.

This table shows that our results agree well with those of Buckman *et al.* in finding no significant alteration in the phosphorus content of the blood in cancer. There are very large variations from the normal average figures, both among the control pathological cases and the cancer cases, but if we take the mean of a large number (which, if Gröbly is correct, should quite definitely show a much larger "phosphorus quotient" for the cancer cases), we find the average quotient in the two series to be practically the same. We have not infrequently obtained high "phosphorus quotients" in cases of cancer, but these results are due entirely to the concurrent anæmia. Normal quotients were obtained in all cases of cancer in which there was no anæmia, and on the other hand, in cases of anæmia in which there was no malignant growth the quotient was usually raised. High quotients are also to be found in other non-malignant conditions, *e.g.* nephritis, jaundice, in which the inorganic or the lipin phosphorus of the plasma is much above normal.

If the figures of Gröbly and of Zerner are further examined, it will be seen that their total phosphorus figures, whether for controls or for cancer cases, are markedly higher than our own. Zerner's *average* phosphorus figure for cancer cases is 28% higher than the *highest* recorded either by Buckman *et al.* or by ourselves, and is 49% higher than our average figure.

Although very unlikely, the high P values obtained by the German authors might have been due to their having used Neumann's macro-method for total P determination, whereas Buckman *et al.* employed Bloor's micro-method, and we the micro-method described in Part I of this series, which gives almost theoretical figures with known amounts of P.

To satisfy ourselves that this was not the explanation of the discrepancy, we have determined the total phosphorus, using Neumann's method and our own side by side, on four different samples of human blood.

TABLE III.—*Neumann's Macro-method for Total P Compared with the Micro-method.*

Blood sample.	Neumann's method.	Micro-method.
Diabetic	31·9	32·3; 32·6; 32·3
,,	33·9	36·5; 36·8; 36·2
Normal	38·3; 37·5	38·0; 38·0; 37·5
,,	36·7; 35·2	36·9; 37·1; 37·1
Average	**35·6**	**36·0**

A control solution of KH_2PO_4 containing 45·0 mgm. P per 100 c.c. gave, by Neumann's method, 44·1; 44·7 mgm. P per 100 c.c.

Table III shows that the two methods give the same values, within a small experimental error. It is therefore not possible to explain the very high total phosphorus values found by the German investigators for whole blood,

both of normals and of cancer cases, on the grounds of differing analytical procedure.

In any case, a laboratory method for the diagnosis of cancer which does not distinguish cases of malignant growth from such common conditions as simple anæmia and catarrhal jaundice—conditions in which the differential diagnosis from malignant disease frequently arises—can have little value, and may even be definitely misleading.

B. THE PHOSPHORUS DISTRIBUTION IN ANÆMIA, POLYCYTHÆMIA AND LEUKÆMIA.

In the first paper of this series it was demonstrated that the ester phosphorus of normal blood is present almost entirely in the corpuscles, and is in amount directly proportional to the percentage volume of corpuscles, so that the quotient of the two quantities forms a constant index which we called the phosphorus index. In the second paper variations in the blood phosphorus distribution and in the phosphorus index were recorded in nephritis, in the later stages of which disease severe anæmia was frequently observed. The present section deals with the phosphorus distribution in the blood in certain other conditions in which the corpuscular volume is markedly changed, namely in simple secondary anæmia, secondary anæmia of malignant disease, pernicious anæmia, polycythæmia and leukæmia.

The methods used are in the main those described in the first paper, with a few modifications in cases where the anæmia was severe. In such cases it is found advisable to use 0·5 c.c. of blood, instead of 0·3 c.c., for the total P determination, and to use larger volumes of protein-free filtrate for the quantitative hydrolysis by phosphatase. We have determined the free, total acid-soluble, hydrolysable and non-hydrolysable, lipin and total P directly on each sample of blood, together with the percentage of corpuscles (hæmatocrit) and the red cell count.

The detailed protocols of 38 pathological and 31 control cases are summarized in Table IV.

TABLE IV.

Disease.	P in mgm. P per 100 c.c. blood.							Red cells in millions per c.mm.	Organic P index.	Hæmatocrit on red cells.	Ratio, hydrolysable : non-hydrolysable.
	Inorganic.	Total acid-soluble.	Ester.	Enzyme hydrolysable.	Non-hydrolysable.	Lipin.	Total.				
Erythræmia (one case) .	3·0	43·0	40·0	11·4	28·6	12·2	55·2	8·7	58	72	1 : 2·5
Secondary anæmia (mean of 9)	3·2	1[illegible]·8	14·6	4·3	10·3	11·2	28·3	3·1	64	22·7	1 : 2·4
Anæmia of malignant disease (mean of 10)	3·2	22·7	19·5	6·2	13·3	13·1	35·5	3·85	65·6	30·0	1 : 2·1
Anæmia of advanced nephritis (mean of 7)	10·3	23·9	13·6	7·2	6·4	11·2	35·2	...	69	18·2	1 : 0·9
Pernicious anæmia (mean of 4)	4·1	14·9	10·8	2·5	8·3	7·6	22·5	1·62	86	13·4	1 : 3·3
Leukæmia (mean of 7) .	3·4	29·3	24·3	9·1	15·2	15·9	45·7	...	140	21	1 : 1·7
Normal (mean of 31) .	3·0	25·7	22·7	6·7	16·0	12·0	37·3	5·2	53	43	1 : 2·4

Commentary.

Erythræmia.—In the single case we have investigated the free phosphate is normal, the acid-soluble phosphorus is increased proportionally to the rise in the relative volume of red cells, and the ratio of enzyme-hydrolysable ester phosphorus to non-hydrolysable ester phosphorus is unchanged. The phosphorus index is within normal limits. Hence (at least in this one case) the red cells, though greatly increased in quantity, seem normal as far as their ester phosphorus analysis is concerned.

Secondary anæmia.—This group contains cases of varying origin, *e.g.* anæmia following hæmatemesis due to gastric ulcer, anæmia associated with hæmorrhoids, with ulcerative colitis, with menorrhagia, with plumbism. The individual cases vary somewhat in their phosphorus partition, but the average figures show that on the whole the changes in P partition are due merely to change in the relative proportion of red cells, and are not due to any qualitative change in the nature of their phosphorus compounds. The slightly higher phosphorus index is in agreement with what one might expect if there were an increased proportion of young cells whose phosphorus content were slightly above normal among the erythrocytes. The ratio of enzyme-hydrolysable to non-hydrolysable ester phosphorus is about normal.

Anæmia of malignant disease.—As far as the phosphorus partition is concerned, the changes are quite similar to those found in secondary anæmia. There is no evidence of any marked increase in the quantity of phosphorus present in the red cells. The mean lipin phosphorus is slightly above normal, since two cases are included exhibiting the lipinæmia of obstructive jaundice associated with carcinoma of the pancreas.

Anæmia of advanced nephritis.—The changes shown here, which have been discussed in some detail in a previous paper (Byrom and Kay, 1927), are entirely different from those of secondary anæmia or the anæmia of malignant disease. Apart from the large increase in inorganic P in advanced nephritis, the ratio of hydrolysable to non-hydrolysable ester P is completely altered. It is of interest to note that the Gröbly quotient is in these cases usually between 3 and 5, *i.e.* these patients would, by Gröbly's criterion, all be suffering from malignant growths.

Pernicious anæmia.—There is here a marked increase in the phosphorus index, *i.e.* a larger content of ester P in 100 c.c. cells. Price-Jones (1910) demonstrated that in this disease the red cells are large, the average volume of a cell according to Haden (1925) being 1·42 times greater than the normal average volume. Larger cells indicate a smaller surface area per unit volume of cells, and a smaller total volume of the surface film with a relatively larger volume of those constituents which do not occupy the surface. There is thus the suggestion that the phosphoric esters are *not* on the surface of the cells, but in the interior, which is the opposite of the distribution of hæmoglobin indicated by the experiments of Bürker (1922) and Emmons (1927), who believe that this pigment is distributed superficially over the cell. The phosphoric esters, however, resemble hæmoglobin in the reluctance with which they leave the corpuscles, little short of laking the blood being sufficient to bring them out into the plasma (Kay, 1926).

Pernicious anæmia is the only condition met with in over 150 cases in which the lipin phosphorus is consistently markedly subnormal. This is explicable partly by the low red cell count (the concentration of lipin P is twice as high in the red cells of normal blood as in the plasma), but there appears to be another factor concerned—possibly the low total area of the red cells, relative to an equal volume of normal erythrocytes. [Gorter and Grendel (1925) have stated that the surface of the red cells of the blood is covered with a film of lipin molecules standing two deep.]

Leukæmia and the phosphorus of the leucocytes.—The mean figures shown for cases of leukæmia in Table IV have, for the purpose of comparison with the other groups in that table, little real significance. This is because the leucocytes present in abnormally large amounts in this group of diseases contain, per 100 c.c., far more phosphorus than the red cells. This we have shown by direct analysis. Therefore the organic phosphorus index, based either on the red cell or on the total cell hæmatocrit reading, is greatly increased above normal.

In two cases of leukæmia the leucocytes have been obtained free from serious contamination with red cells or plasma by centrifuging oxalated blood in a high-speed centrifuge, followed by direct pipetting off of the layer of white cells from between the plasma and the corpuscles. Microscopic examination of a film prepared from this layer shows relatively few red cells, and on mixing a few drops with water, the resulting suspension is almost colourless. A definite volume of white cells is mixed, for convenience in manipulation, with an equal volume of 0·9% NaCl, and a complete phosphorus analysis carried out on the suspension.

The results are shown in Table V:

TABLE V.

Source of leucocytes.	P to nearest mgm. per 100 c.c. cells.						
	Inorganic.	Total acid-soluble.	Ester.	Enzyme hydro-lysable.	Non-hydro-lysable.	Lipin.	Residual (nucleic acid or nucleo-protein)
1. Blood from case of chronic myeloid leukæmia	6	90	84	41	43	60	...
2. Blood from case of chronic lymphatic leukæmia	10	82	72	44	28	64	111
3. Blood from case of acute myeloid leukæmia	10	69	59	...	...	75	...
4. Three approximately normal bloods (average)	20	99	79	56	23	...	. .
Red cells (normal) . . .	3	56	53	16	37	18	0

The values in cases 1 and 2 have been directly determined; case 3 gives figures obtained from a sample of leukæmic blood in which the plasma, whole blood and red cell phosphorus partition have been determined separately, and

the leucocyte figures calculated from these values and from the relative volume of white cells shown by a high-speed hæmatocrit. Row 4 gives average figures for the leucocytes in three samples of approximately normal human blood. The leucocytes could not, in these cases, be pipetted directly from the centrifuged blood, but were obtained by separation of as much as possible of the red cell and plasma layers with a pipette, re-suspending the leucocyte layer in plasma and again centrifuging, when the leucocyte layer could be lifted from the remaining red cells almost completely, drained for a short time on hard filter-paper, and suspended in saline. It is clear that this latter method is far less accurate than that of direct separation just described. Possibly the high inorganic phosphorus may be due to enzymic autolysis of part of the phosphoric esters of the white cells, which we have found to contain a very active phosphatase. It will be observed that the leucocyte lipin P is very high.

According to Gröbly's criterion, all our leukæmia cases (with the exception of one case in which the leucocyte count had been reduced below normal by treatment with X-rays) would have been classified as harbourers of malignant growths.

SUMMARY.

1. In malignant disease unaccompanied by anæmia there is no significant change, as compared with non-anæmic non-cancerous controls, in the amount of any of the following kinds of phosphorus compounds in the blood: free phosphate, phosphoric esters hydrolysed by phosphatases, phosphoric esters not hydrolysed by phosphatases, lipin phosphorus, total phosphorus.

2. In malignant disease accompanied by anæmia the changes which occur in the above blood constituents are entirely explicable on the basis of the anæmia, and are in no way specific for cancer.

3. The determination of the "phosphorus quotient" as described by Gröbly is of no diagnostic value in this disease.

4. In severe obstructive jaundice, such as that accompanying carcinoma of the pancreas, the lipin phosphorus of the blood is markedly increased.

5. In polycythæmia and simple secondary anæmia, as in the anæmia of carcinoma, the changes from the normal in the blood phosphorus distribution are the result merely of a change in the relative proportion of the red cells to plasma. This is not the case in the anæmia of advanced chronic nephritis or in pernicious anæmia; in both these diseases the qualitative and quantitative composition of the cell with regard to phosphorus compounds appears to be changed.

6. Leucocytes are relatively rich in phosphorus compounds, a given volume of leucocytes containing two to three times the quantity of phosphorus as the same volume of red cells. In leukæmia, therefore, phosphorus determinations on the whole blood vary with the severity of the anæmia and the leucocytosis in an irregular manner.

The expenses of this work have been defrayed by grants from the Research Fund of the London Hospital Medical College.

REFERENCES.

BUCKMAN, T. E., MINOT, G. R., DALAND, G. A., AND WELD, M.—(1924) *Arch. Int. Med.*, **34**, 181.
BÜRKER, K.—(1922) *Arch. Neerland. de Physiol.*, **7**, 309; quoted from C. Lovatt Evans, 'Recent Advances in Physiology.'
EMMONS, W. F.—(1927) *J. Physiol.*, **64**, 215.
GORTER, E., AND GRENDEL, F.—(1925) *J. Exp. Med.*, **41**, 439.
GRÖBLY, W.—(1921) *Arch. f. klin. Chir.*, **115**, 261.
HADEN, R. L.—(1925) *Folia Hæmatologica*, **31**, 113.
KAY, H. D.—(1926) *Brit. J. Exper. Pathol.*, **7**, 177.
Idem., AND BYROM, F. B.—(1927) *Ibid.*, **8**, 240 and 429.
PRICE-JONES, C.—(1910) *Brit. Med. J.*, **2**, 1418.
VORSCHÜTZ, J., AND VORSCHÜTZ, J.—(1922) *Deutsch. med. Wochenschr.*, **48**, 861.
ZERNER, H.—(1924) *Ztschr. f. Krebsforschung*, **21**, 157.

I. THE ACCURATE MEASUREMENT OF THE PROPORTION OF CORPUSCLES AND SERUM IN BLOOD.—II. THE VOLUME OF RED BLOOD-CORPUSCLES IN VENOUS AND OXYGENATED BLOOD AND AFTER EXPOSURE TO VARIOUS SATURATIONS OF CARBON DIOXIDE.

FREDERICK HORACE SMIRK.

From the Laboratory of Clinical Investigations and Research, Manchester Royal Infirmary.

Received for publication January 21st, 1928.

I. THE ACCURATE MEASUREMENT OF THE PROPORTION OF CORPUSCLES AND SERUM IN BLOOD.

IN the study of changes reported to take place in the size of corpuscles when the tension of carbon dioxide in the blood is altered, and in the closely related problems of chlorine interchange between corpuscles and serum, any hæmocrit measurements must be made in the absence of air, so that the tension of blood gases may be unchanged.

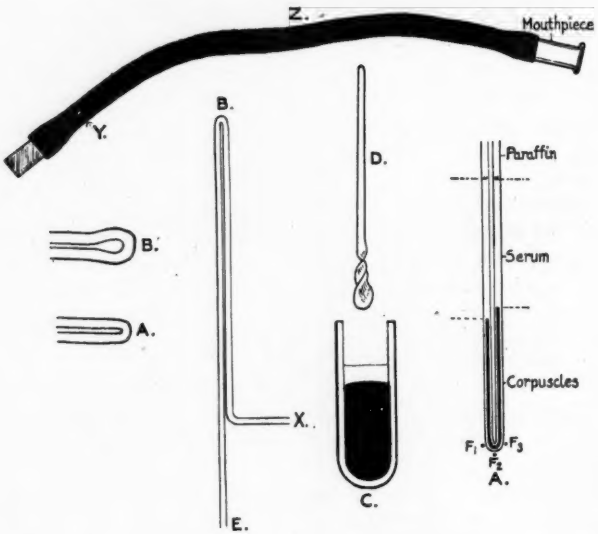
The method described fulfils this condition, and depends on centrifuging small quantities of blood which have been drawn into capillary U-tubes between layers of paraffin. In this way blood may be transferred from a vein to the tubes and centrifuged without contact with air. 4 or 5 tubes may be filled in 2 mins., and later centrifuged in the same "carrier" tube.

Experimental Details.

A piece of glass tubing 1 cm. diameter is drawn into a capillary about 8 ft. long and broken into sections of 7 in. These are bent in a flame about 2 mm. high, using a micro burner, to the shape EBX indicated on the diagram. The capillary limbs should lie parallel at the bend, which should be sharp as in "A," not rounded as in "B."

If the capillary tubes are bent with only the outer margin of one side in the micro-flame the procedure is easy. The tube is filled by attaching the end "X" to the slit "Y" in the rubber tube "Z," whose distal end is blocked by a short length of solid glass rod.

Blood is collected by a syringe and needle in which the air space has been replaced by sterile paraffin, and is delivered to the bottom of a tube "C" containing a little potassium oxalate under paraffin. The blood is thoroughly mixed with oxalate by rotation of the glass propeller "D." The opening "E" is now dipped into the paraffin and ½ in. of this is drawn into the tube. Approximately 4 in. of blood are now drawn in, followed by another ¼ in. of paraffin.



The hæmocrit tube is removed from the protective layer of paraffin, and the blood and paraffin are slowly sucked round the bend until there is equal blood on either side. The limbs are cut off about ¼ in. above the blood, and the capillary U-tube placed in a small test-tube with a thickened base made to fit the hæmocrit.

The samples are centrifuged at three or four thousand revolutions per minute for half an hour or until constant volume.

After centrifugalization the U-tubes are placed on a sheet of paper, and pricks made exactly opposite the levels of corpuscles and serum in both limbs and round the bend of the tube. A very approximate correction can be introduced for the bend by taking three points, F_1, F_2, F_3, and adding the measurements F_1F_2, F_2F_3 to the lengths of their respective columns. Measurements should be guessed to as near 0·1 mm. as is possible, using a pair of fine dividers for this purpose. The percentage of corpuscles is readily calculated.

It will be seen that several observations can be easily, readily and cheaply made on various samples of blood, and all the capillary U-tubes may be centrifuged together. The difficulties in cleaning the thick graduated capillary tubes ordinarily used are avoided, and the tendency to leakage at high speed

to which these tubes are subject is overcome by a method which does not admit of leakage.

Results.

On three separate samples of blood, *A*, *B* and *C*, which were centrifuged together for the same time at the same speed:

Sample A:		Sample B:		Sample C:	
47·7		50·4		47·8*	
47·9		50·7		46·3	
47·2	Average 47·4.	50·8	Average 50·4.	47·2	Average 46·7.
47·2		52·2*		46·6	
47·1		49·9		46·7	

* Aberrant value.

Errors Introduced by the Capillary Tube.

1. *Due to irregularity of the tube.*—It will be seen from the above results that although most are in close agreement, about one in four or six show a relatively marked deviation. These results should be ignored—they are the results of irregular tapering of capillary tubes.

2. *Due to the nature of the glass.*—If the capillary tubes have an internal bore of 0·33 mm., then 10 c.c. of blood in such tubes would be exposed to a surface of about 590 cm., whereas in a test-tube of about 3 cm. diameter it would only be exposed to a surface of 13 sq. cm.

It would therefore seem necessary to determine whether the use of ordinary glass was prejudicial to the results.

Venous blood in capillary tubes of ordinary and Jena glass was allowed to stand three hours before centrifuging. The percentage of corpuscles was then determined and averages of each set of tubes taken.

Results.

Average of 5 Jena glass tubes	47·7
„ 5 ordinary glass tubes	47·3

The above experiment was then repeated using a larger number of capillary tubes, some of which were allowed to stand for 2½ hours and some for 5½ hours before centrifuging.

After 2½ hours' exposure:

Average of 4 Jena glass tubes	50·47
„ 4 ordinary glass tubes	49·7

After 5½ hours' exposure:

Average of 5 Jena glass tubes	50·0
„ 4 ordinary glass tubes	48·9

These changes probably indicate that the alkali of the ordinary glass causes an increase in the pH of the blood and a slight change in the

corpuscular volume. Where the tubes are spun in the hæmocrit almost immediately after filling this error is negligible. That carbon dioxide is not alone responsible for variations in cell volume has been demonstrated by Evans (1925).

Errors Introduced by Incomplete Separation of Corpuscles and Serum.

Errors due to capillary tube defects are not difficult to overcome, but other factors, commonly ignored, are in my opinion responsible for many discrepancies between results obtained by hæmocrit and other methods.

The main error appears to be incomplete separation of corpuscles, and in consequence a difference in results by macro-centrifugalization and hæmocrit readings. The following experiments were designed to study some of these factors.

About 4 c.c. of a sample of oxygenated blood were placed in a graduated centrifuge tube, and a series of small U-tubes of different lengths, used for hæmocrit determinations, was filled from another portion of the same sample, placed in the remaining cups of the centrifuge, and the whole spun at about 4200 revolutions per minute for twenty minutes.

Results.

Large centrifuge tube readings:

Total volume	3·80 c.c.
Corpuscular volume	2·02 „
∴ Corpuscular percentage	53·3%

Hæmocrit tube readings:

Length of blood column in capillary tube.	Estimated percentage of corpuscles.
6·85 cm.	50·0
6·70 „	49·3
6·02 „	48·8
5·66 „	48·6

This suggests that at the same speed of rotation there is more complete separation in capillary columns than in larger masses of blood, and that separation is more complete in shorter capillary columns. This latter error, however, is readily eliminated if longer periods of centrifugalization are used.

The sample of corpuscles obtained by macro-centrifugalization was mixed thoroughly and spun in the hæmocrit at a speed of 6300 revolutions per minute. A sample of the original blood was also centrifuged in this way.

Corpuscular mass when spun in the hæmocrit showed:

Percentage of corpuscles.	Percentage of serum.
Average of 3 readings 91·2	8·8

Whole blood when spun in hæmocrit showed:

Percentage of corpuscles.	Percentage of serum.
Average of 3 readings 47·6	52·4

The way in which this affects biochemical investigations may be illustrated by estimating the percentage iron in whole blood and in the corpuscular mass, which has already been shown to contain 8·8% of serum. It will be possible to illustrate by this means how two values of the corpuscular blood iron in close agreement may be arrived at by two different methods and yet contain the same error.

The iron estimations were made by the method described by Smirk (1927).

Results.

Corpuscular blood-iron . . . . . 122·0 mgm.
Whole blood-iron . . . . . 63·0 ,,

The sample of corpuscles has been shown to contain 8·8% of serum, so that the corpuscular blood-iron of a completely centrifuged sample would be—

$$\frac{122{\cdot}0 \times 100}{100 - 8{\cdot}8} = 133{\cdot}8 \text{ mgm.}$$

The whole blood-iron is 63·0 mgm., and its corpuscular percentage 47·6. By calculation the corpuscular blood-iron is—

$$\frac{63{\cdot}0 \times 100}{47{\cdot}6} = 132{\cdot}3 \text{ mgm.}$$

These results, 133·8 and 132·3, are sufficiently close. It will be seen, however, that it would be possible by taking the reading on the macro-centrifuge as correct to obtain consistent results on the corpuscular blood-iron by calculation and direct estimation.

For example in this experiment:

Percentage of corpuscles by direct reading on the macro-centrifuge . . . = 53·3
Whole blood-iron . . . . . = 63·0
∴ Uncorrected corpuscular blood-iron by calculation—

$$= \frac{63 \times 100}{53{\cdot}3} = 118{\cdot}2 \text{ mgm.}$$

Uncorrected corpuscular blood-iron by direct estimation . . . . . = 122·0 mgm.

The latter results, though fairly close, are known to be inaccurate, because the corpuscular mass contains 8·8% of serum. The discrepancy of 4 mgm. is due to the removal before mixing of the upper layer of corpuscles which contains most serum. The sample used for estimation was therefore more concentrated than the volume reading would show.

If samples are taken from different levels in the unmixed corpuscular mass the corpuscular iron will vary by 10 or 15 mgm., being greater in samples from the base of the centrifuge tube.

Discussion.

Gram (1921) maintains that blood centrifuged at 3000 r.p.m. for 90 minutes yields results within 0·5% of those by hæmocrit, but Ege (1920) found prolonged spinning at 2000-3000 r.p.m. left 10% of serum in the

corpuscular mass extractable by the hæmocrit. This is supported by Millar (1925), who found that by centrifuging blood to constant volume at increasing speeds he obtained decreasing corpuscular percentages, gradually approaching the level of the true percentage.

It will be realized from this paper that more complete separation of corpuscles takes place in a capillary than in an ordinary centrifuge tube when spun at the same speed for the same time, confirming a conclusion of Rossdale (1923) that a fine capillary is essential for accurate working.

It has been shown that errors resulting from the serum content of a corpuscular mass can be corrected by a hæmocrit observation on the sample of corpuscles.

Rossdale (1923) finds the percentage volume of corpuscles is less in oxalated and citrated blood than in blood untreated by anti-coagulants or prevented from clotting by hirudin. This does not affect the results of this paper, but the amount of oxalate must be carefully standardized if reliable observations are to be made with different samples of blood.

For comparison with other work, the speed of the centrifuge estimated by a bicycle speedometer was found to be 4200 r.p.m., and with the hæmocrit attachment 6300 r.p.m. Unless otherwise stated the samples were spun for 25–30 minutes at these speeds.

In most experiments it was arranged that samples of blood under comparison were spun together, so that the integrity of these results for comparative purposes does not depend on the absolute accuracy of the cell volume determinations.

II. THE VOLUME OF RED BLOOD-CORPUSCLES IN VENOUS AND OXYGENATED BLOOD AND AFTER EXPOSURE TO VARIOUS SATURATIONS OF CARBON DIOXIDE.

Hamburger (1897), and more recently Price-Jones (1920), observed physiological variations in the size of red corpuscles by measuring dried films. It is important to ascertain whether this change is large enough to make hæmocrit observations on venous blood inapplicable to oxygenated samples. Blood was collected from a congested vein and delivered under paraffin. A portion of the well-mixed sample was withdrawn, oxygenated by shaking thoroughly with air, and hæmocrit observations then made on both venous and arterialized blood. The percentage of corpuscles was estimated in each case.

Results.

% Corpuscles in Blood.

	Sample before oxygenation.			Sample after oxygenation.			Difference.
A.	Average of	4	observations = 44·9	Average of	3	observations = 44·3	− 0·6
B.	„	5	„ = 44·2	„	5	„ = 43·7	− 0·5
C.	„	5	„ = 45·5	„	7	„ = 45·3	− 0·2
D.	„	5	„ = 47·7	„	7	„ = 48·0	+ 0·3
E.	„	3	„ = 47·6	„	4	„ = 46·4	− 1·2
F.	„	3	„ = 47·6	„	4	„ = 46·7	− 0·9

The results show a slight decrease in the percentage volume of the corpuscles of arterial as compared with venous blood.

More definite changes take place if blood is exposed to higher tensions of carbon dioxide than are encountered physiologically.

Ten c.c. of oxalated blood were shaken with air and 3 c.c. removed and covered with paraffin. The remaining 7 c.c. were placed in a glass chamber containing 50% CO_2 and 50% air, and the blood shaken or rolled over the walls of the vessel to establish gaseous equilibrium. Samples taken from the chamber after ½ hour, 1½ hours, 2½ hours and 15 hours were centrifuged under paraffin, and the results compared with readings obtained from the original sample.

Results.

Exposure to 50% carbon dioxide produced a marked increase in the percentage volume of corpuscles. The initial average value for corpuscular percentage was 45·4%, and after carbon dioxide the percentage became 50·2 in the first ½ hour, 50·9 in 1½ hours, and 50·4 in 2½ hours. It fell to 49·5 only after 15 hours.

This observation on oxalated human blood exposed to 50% CO_2 differs from the observations of Mellanby and Wood (1923) on sheep's blood exposed to 100% CO_2 in that the altered size of the corpuscles is not markedly a function of time. Evidence was obtained by Mellanby and Wood that no damage to the corpuscles resulted from exposure to this concentration of CO_2, since the volume of corpuscles was restored to normal after oxygenation of the blood.

It was decided to repeat the experiment described above using 100% CO_2, and in addition taking hæmocrit readings on a sample oxygenated after long exposure to this gas.

Results.

The results with 100% CO_2 were substantially the same as with 50% CO_2. The initial volume of corpuscles was 44·8%, and after exposure to 100% CO_2 the averages of 4 or 5 hæmocrit readings taken on each sample became 51·9% in ½ hour, 52·5% in 1½ hours, 51·6% in 3 hours, only diminishing slightly to 51·2% corpuscles in 21 hours.

After 21 hours' exposure to 100% CO_2 the corpuscular volume was restored to 45·9% on desaturation. In this my results agree with Mellanby and Wood (1923), the conclusion being that exposure to 100% CO_2 for 21 hours does not appreciably damage the limiting membrane of the corpuscle.

It is difficult to account for the striking difference in the results on human and sheep's blood. My observations were conducted at room temperature (about 17° C.) on human blood, with strictest precautions to avoid air leakage. Potassium oxalate was used as an anti-coagulant, and all vessels had been cleaned with hot water, distilled water, alcohol and ether and were then warmed and air drawn through by a suction pump.

The use of paraffin to maintain the gaseous content of a blood sample at a constant value is not quite free from objection, as carbon dioxide is definitely soluble in paraffin. 5 out 15 c.c. of carbon dioxide were absorbed from a cylinder of the gas, which was allowed to stand over about 15 c.c. of paraffin in an evaporating dish for 24 hours. About 1 or 1½ c.c. of this were dissolved in the first hour or so.

In view of the results published by Mellanby and Wood (1923), it was decided to repeat the experiments described on a sample of defibrinated sheep's blood.

Results.

A fresh sample of defibrinated sheep's blood had a corpuscular percentage of 36·6 when untreated, and on exposure to 100 % CO_2 this became 40·1 after 20 minutes, 40·4 after 2½ hours and 40·2 after 15 hours. A portion of the same sample was allowed to stand 24 hours and the corpuscular percentage was found to be 36·1. After exposure to 100 % CO_2, as in the previous experiment, the hæmatocrit readings were 40·1 in 20 minutes, 39·2 in 2½ hours and 38·5 in 24 hours.

The results obtained by my technique are therefore seen to be exactly the same for defibrinated sheep's blood as for oxalated human blood.

SUMMARY.

1. An accurate micro hæmocrit method has been described by a procedure which leaves the gaseous tension of the blood unchanged.

2. The centrifugalization of blood in ordinary and capillary tubes does not yield comparable readings of the corpuscular percentage.

3. If a 6-centimetre column of blood is spun on a reliable hæmocrit at about 6000 revolutions per minute for 20 minutes, with double this time for corpuscular masses, the absolute error is small and comparative observations are usually very close.

4. If blood is allowed to stand in capillary tubes of Jena and ordinary glass for two or more hours the percentage volume of corpuscles diminishes slightly in both types of glass, but more so in ordinary glass. This difference is not more than 2% after 5 hours' exposure. It is suggested that this is due to the alkali of glass causing an increase in pH of the blood. This factor would be due to the enormous increase in glass surface relative to the volume of blood.

5. Oxygenation of venous blood from the area causes a slight diminution in the percentage volume of corpuscles. An average of 55 hæmocrit observations shows a variation of rather more than 1% of the volume of corpuscles.

6. Exposure of human blood to 50% CO_2 caused an increase of 11% in the volume of corpuscles, which was maintained within 2% after 15 hours' exposure. 100% CO_2 produced a change of about 17% in the volume of corpuscles, which was maintained within 3% after 21 hours' exposure. Similar experiments on defibrinated sheep's blood produced like results.

7. Complete restoration of the corpuscular volume is obtained if the blood is removed from the carbon dioxide and desaturated.

I am much indebted to Prof. Raper and Prof. Craven Moore for their kind interest and helpful criticism, and to the Medical Research Council, who have defrayed the expenses of this research.

REFERENCES.

EGE, R.—(1920) *Biochem. Ztschr.*, **109**, 241.
EVANS, C. A. LOVATT.—(1925) 'Recent Advances in Physiology,' London.
GRAM, H. C.—(1921) *Compt. Rend. Soc. Biol.*, **84**, 151.
HAMBURGER, H J.—(1897) *Ztschr. f. Biol. München*, **35**, 252.
MELLANBY, J., AND WOOD, C. C.—(1923) *J. Physiol.*, **57**, 113.
MILLAR, W. G.—(1925) *Quart. J. Exper. Physiol.*, **15**, 187.
PRICE-JONES, C.—(1920) *J. Path. & Bact.*, **23**, 371.
ROSSDALE, G.—(1923) *Quart. J. Med.*, **16**, 245.
SMIRK, F. H.—(1927) *Biochem. J.*, **21**, 36.

THE STRUCTURE OF THE *V. CHOLERAE*, WITH REFERENCE TO ITS IMMUNIZING PROPERTIES.

R. W. FAIRBROTHER,

Research Fellow, Lister Institute; Late Dickinson Research Scholar, Manchester.

From the Pasteur Institute, Paris, and Lister Institute, London.

Received for publication March 1st, 1928.

IT is a generally accepted fact that cholera vaccines are of great prophylactic value, but in spite of the many different methods of preparation which have been used, not one is free from one or other objection. The immunity produced by such an inoculation is believed to be of comparatively short duration—some 6 to 12 months—and as in cholera districts re-inoculations are frequently necessary, it is obvious that a vaccine producing a good degree of immunity with the minimum of reaction is very desirable. Horder and Ferry (1926) have described a substance of high agglutinogenic value and practically non-toxic, which was easily obtained in washings of the ectoplasm of organisms. Ferry and Fisher (1924) had prepared a similar substance from a number of micro-organisms—*B. typhosus*, *B. coli*, *B. paratyphosus A* and *B*, pneumococcus, streptococcus, *B. pertussis* and gonococcus—and compared it by serological examination with antigens prepared by other methods. The results showed that the substance obtained was highly agglutinogenic and of very low toxicity for animals. Such a vaccine, if it could be shown to have corresponding protective value, would be ideal for cholera prophylactic purposes. The following experiments were performed with a view to investigating the value of such a substance, prepared from the *V. cholerae*, with regard to its ability to protect guinea-pigs against a lethal dose of the same organism, since it is an open question whether the estimation of antibodies *in vitro* serves as a true index of such protection.

Survey of Cholera Vaccines.

A large number of different methods have been employed in the preparation of cholera vaccines, and only a brief account of the main types will be given here.

1. *Living vaccines :*

(*a*) Ferran (1885), in Spain, inoculated subcutaneously a small amount of a virulent fresh culture.

(*b*) Haffkine (1893) modified this on Pasteurian ideas and gave an attenuated living culture subcutaneously for the first dose, followed by an exalted culture. Later (1896–1909) the exalted culture was used alone. Extensively employed in India with good results but rather severe reaction.

2. *Ordinary killed cultures :*

(*a*) Wright and Bruce (1893) suggested that the addition of 0·5% carbolic acid to Haffkine's vaccines would render them less powerful but safer.

(*b*) Heat at 56°–60° C. 1 hour has been universally used (Dopter, 1915 ; Cantacuzene, 1920 ; etc.).

(*c*) Castellani and Mendelson (1915) combined it with T.A.B. vaccine, giving " tetravaccines."

(*d*) Vincent (1915) employed ether, which killed the vibrios and also extracted the toxic lipoid substances.

3. *Sensitized vaccines :*

(*a*) These have been employed to a large extent in Japan, the idea being to decrease the reactions. The advantages and disadvantages of this method of preparation have been discussed fully by Tahano, Ohtsubo and Inouye (1926).

(*b*) Miura (1920) dried a sensitized vaccine in order to increase its stability.

4. *Bacterial extracts :*

(*a*) Strong (1907) used a saline suspension of a young agar culture, heated 60° C. 1 hour, then placed at 37° C. for 3–4 days and finally filtered.

(*b*) Besredka and Golovanoff (1923) obtained their " antivirus " by incubating a broth culture 8–10 days at 37° C., filtering, inoculating the filtrate with the same organism and incubating 37° C. 8 days. This second filtrate gave rise to a degree of protection, without the formation of antibodies, in guinea-pigs.

5. *Bilivaccine :*

(*a*) Glotoff (1923) applied Besredka's bilivaccine, sensitizing with bile and administering the vaccine orally. He obtained protection in rabbits by such a method. Now used extensively in Russia and India (Besredka, 1927).

From the above list it will be seen that no satisfactory form of cholera vaccine has been established ; the general idea since the original Ferran campaign has been to obtain a form which will reduce local and general reaction without interference with the production of immunity. In so far as heat- or phenol-killed cholera vaccine is concerned, war experience showed

that doses of 5000 million gave rise to little local or general disturbance. However, the attempts to obtain the cholera receptors without the toxic substances of the bacterial cells have usually resulted in some decrease in protective properties. Fox (1916), in an experiment on rabbits to find the relative values of various kinds of vaccines combining high immunizing properties, as estimated by the production of agglutinins, with the minimum of reaction, as gauged by the body temperature and degree of swelling at the site of inoculation, found that the carbolized vaccine was the best. The substances producing immunity would appear to be intimately connected with the bacterial protoplasm. Levaditi and Mutermilch (1908) stated that the cholera antigen was soluble in slightly dilute alcohol (85 %), and was not related to either the lipoids of the bacterial body or the albuminous substances precipitated by heat. Recent experiments of Landsteiner and Levine (1927) indicate that in the *V. cholerae* there is an antigenic complex of a protein and a specific substance, which is possibly a carbohydrate.

TECHNIQUE.

The strain of *V. cholerae* used throughout this work was one received at the Pasteur Institute, Paris, from India. By passage, intraperitoneally, through a number of guinea-pigs a state was reached when 1/20 of an agar culture (24 hours) intraperitoneally would kill a guinea-pig of 250 gm. in less than 24 hours; the strain exhibited a great tendency to lose its virulence, but by preservation in an ice-chest and subsequent animal passage the above degree of pathogenicity was maintained fairly constant, and was tested by animal inoculation before each experiment.

Preparation of the centrifugate.—Owing to the great motility of the *V. cholerae* and the slowness of the centrifuge—6–7,000 per minute—as compared with the speed of a Sharples centrifuge which the American workers used, the greatest difficulty was experienced with the centrifuge available in obtaining a sterile centrifugate, and after several attempts the following method was employed: A 24 hours' culture in bouillon (pH 8·3) was placed in the centrifuge and spun for 2 hours. The upper supernatant fluid was collected and re-spun for 1 hour, when the upper portion was collected and 0·5 % phenol added; this constituted the "centrifugate." This was a clear fluid, but contained, however, a number of organisms, approximately half a million per c.c.; thus it was not as pure a centrifugate as that employed by Ferry and Fisher in their experiments with other organisms, but it contained all the easily detached substances which they termed the ecto-antigen and was the main antigen of the organisms they examined. This substance was prepared before each series of experiments, and where comparison was made with vaccines prepared by other methods the same culture was used for each, in order that there should be no differences in the antigenic strength of the culture.

The other vaccines employed were prepared by heating the whole bouillon cultures in a water-bath to 100° C. or 56° C. for varying times, and afterwards adding 0·5% phenol.

The test animals were guinea-pigs, and so far as could be arranged the different experiments were performed with animals of approximately the same

weight. The lethal test-dose, which was determined on fresh animals before each experiment, was always administered intraperitoneally.

EXPERIMENTAL.

Experiment I.—The centrifugate and, as a control, ordinary sterile bouillon, were inoculated intraperitoneally in doses of 0·5, 1 and 2 c.c. on alternate days; 14 days after the last dose they and 4 fresh animals were given the test dose (1/20 agar 24-hours culture) intraperitoneally.

Inoculation.	Number employed.	Number survived.	% survival.
(*a*) Centrifugate	7	6	86
(*b*) Normal bouillon	3	1	33
(*c*) *Nil* (controls)	4	2	50

A certain degree of protection appeared to be present in the animals inoculated with the centrifugate, but this experiment was far from conclusive, the test dose being rather too small. The possibility of a local reaction could not be excluded as all the inoculations were performed intraperitoneally. Pfeiffer and Issaeff (1893) showed that protection by non-specific substances could be obtained against an intraperitoneal lethal dose of *V. cholerae* when the interval between immunization and test inoculation was very short.

A further series was inoculated with similar doses of vaccine and normal bouillon, but subcutaneously, followed 19 days after the last dose by the test dose intraperitoneally (1/16 agar culture, 24-hours).

Inoculation.	Number employed.	Number survived.	% survival.
(*a*) Centrifugate	3	3	100
(*b*) Bouillon	3	0	0
(*c*) *Nil* (controls)	3	0	0

Although the number of animals used in this experiment was small, the results were much more conclusive. A definite protection was present in those animals inoculated with the centrifugate; the time between the last inoculation and the lethal dose did not suggest that this protection was of a non-specific nature. The question now arose wherein lay the value of the vaccine. It has been stated that the centrifugate contained the "ecto-antigen" and some vibrios; in addition there would be present the autolysed products of dead organisms, although not in very large amount in a young culture. With a view to further determining the active factor, the following experiments were performed:

Experiment II.—From the same 24-hours bouillon culture the following fractions were prepared:

(*a*) A vaccine made of the whole culture heated 56°C. 1 hour.

(*b*) A centrifugate prepared as above.

(*c*) The sediment after the first spinning, made up to the original volume by the addition of 0·85% NaCl, emulsified and heated to 56°C. 1 hour.

To each of these 0·5% phenol was subsequently added. Three doses of each, 0·5, 1 and 2 c.c., were given subcutaneously on alternate days, and 9 days after the last dose a massive test-dose* was given intraperitoneally.

Inoculation.	Number employed.	Average weight.	Number survived.	% survival.
(*a*) Whole vaccine . . .	8	248 gm.	6	75
(*b*) Centrifugate . . .	7	260 ,,	3	43
(*c*) Bacterial deposit . .	5	240 ,,	4	80
(*d*) *Nil* (controls) .	4	240 ,,	0	0

The greatest protection was present in those animals which had received the actual bodies of the organisms; this is most marked where the animals have received inoculations of vibrios from which the ecto-antigen had been removed, *i.e.* those given the bacterial deposit. A certain amount of protection, however, is present in those guinea-pigs which were given the centrifugate; this is much less than in the other two cases, and in view of the high degree of protection acquired by giving the bacterial deposit, it would seem that the vibrios present in the centrifugate were responsible mainly for the protection produced.

Experiment III.—As it was found impracticable to obtain the centrifugate entirely free from the *V. cholerae*, a rapid filtration was performed through a Chamberland bougie; this was done rapidly in order to exclude, as far as possible, the removal by absorption of the soluble immunizing substances by the filter. The filtrate was preserved by the addition of 0·5% phenol, and was inoculated subcutaneously and compared with a vaccine (56°C., 1 hour) also carbolized to 0·5%; the doses were 0·5, 1 and 2 c.c. on alternate days, and 10 days after the last dose the test dose (1/16 agar culture, 24-hours) was given intraperitoneally.

Inoculations.	Number employed.	Average weight.	Number survived.	% survival.
(*a*) Vaccine 56°C. 1 hour .	4	250 gm.	4	100
(*b*) Filtrate	3	285 ,,	1†	33
(*c*) *Nil* (controls) . .	3	295 ,,	1†	33

The results obtained were very interesting, for while a good protection was obtained following the inoculation of the whole vaccine, a very small degree of protection, if indeed any, was afforded by the filtrate, as the rather large weight of the surviving animal might have been the responsible factor; only one of the latter animals and one of the control animals survived, and both these were large animals. By comparing the above three experiments, evidence can be obtained that protection in guinea-pigs against cholera cannot be produced to any marked degree by the inoculation of the centrifugate. The last two experiments indicate that the protection obtained in the previous experiments by the centrifugate was mainly the result of the presence of actual vibrios in the fluid.

* Test dose (*a*), (*b*) and (*c*), 1/10 agar slope (24 hours); (*d*), 1/15 agar slope (24 hours).

† The animals which survived in Groups (*b*) and (*c*) were the largest in the experiment and each weighed 310 gm.

Experiment IV.—This experiment was performed in order to test the protective powers of vaccines heated at 100° C. It is a well-known fact that heating a vaccine to 100° C. destroys some of its antigenic properties, this being particularly marked with motile organisms, when the heat-labile factor which is attributed to the flagellar portion of the protoplasm is destroyed.

Vaccines were prepared from a bouillon culture (24 hours), one heated at 56° C. 1 hour, and the other 100° C. 1 hour; afterwards both were carbolized 0·5%. 0·5, 1 and 2 c.c. were inoculated subcutaneously on alternate days, followed 9 days after by a lethal dose (1/12 agar culture, 24 hours) intraperitoneally.

Inoculations.	Number employed.	Average weight.	Number survived.	% survival.
(*a*) Vaccine 56° C. 1 hour	4	350 gm.	4	100
(*b*) „ 100° C. 1 „	4	325 „	4	100
(*c*) *Nil* (controls)	7	435 „	3	43

Definite protection was obtained by the use of both vaccines; the control animals were much larger than the others.

A further experiment was performed in which the lethal dose was increased to 1/10 agar culture, 24 hours.

Inoculations.	Number employed.	Average weight.	Number survived.	% survival.
(*a*) Vaccine 56° C. 1 hour	8	300 gm.	2	25
(*b*) „ 100° C. 1 „	8	290 „	5	62·5
(*c*) *Nil* (controls)	4	270 „	0	0

The process of heating the vaccine to 100° C. for 1 hour did not in any way appear to affect its power to protect guinea-pigs; in this case it appeared to have enhanced it somewhat.

The following experiment is given here as a further illustration of the absence of good protection following the inoculation of the centrifugate. The above experiments prove that the substance giving rise to protection in animals is heat-stable. Here an attempt was made to produce protection by means of the centrifugate heated to 100° C. for 1 hour; 0·5, 1 and 2 c.c. were given subcutaneously on alternate days, followed 14 days later by a large lethal dose (1/10 agar culture, 24 hours).

Inoculations.	Number employed.	Average weight.	Number survived.
(*a*) Centrifugate 100° C. 1 hour	4	300 gm.	0
(*b*) *Nil* (controls)	4	280 „	0

No animals survived; this further experiment increases the evidence that the centrifugate is much inferior to the ordinary heat-killed vaccine in the power to afford protection to guinea-pigs against a lethal dose of *V. cholerae*.

Summary of the experiments.—A small degree of protection was obtained by the use of the centrifugate, but this was probably due to the presence of *V. cholerae*. The substance giving rise to protection in animals was heat-stable.

DISCUSSION.

The test of efficiency of the various vaccines prepared from *V. cholerae* in this investigation has been the protection afforded against a lethal intraperitoneal dose. Several objections have been raised against such a procedure, one very important one being that cholera peritonitis of guinea-pigs is an entirely different condition from human intestinal cholera; however, the fact that the same method of examination has been utilized to test the efficiency of the earlier vaccines with comparatively good results and has also been used in *B. typhosus* experiments on prophylaxis is considered a sufficient justification for such a method of investigation.

The results obtained show that the centrifugate prepared from *V. cholerae* had very little power of inducing immunity in guinea-pigs, and that what protection occurred was probably the result of the vibrios present even after prolonged centrifugalization. How can these results be reconciled with those obtained by Ferry and Fisher, who found that this substance was highly antigenic in so far as antibody development was concerned? In the case of certain cocci there is much evidence to show that immunity can be obtained by the soluble fraction. Perlzweig and Keefer (1925), for example, obtained and purified a soluble immunizing fraction of the pneumococcus from the filtrate. In dealing with motile organisms, such as *B. coli*, *B. typhosus* and *V. cholerae*, however, another factor must be considered: Smith and Reagh (1903) stated that there were two factors in the agglutinability of the hog cholera bacillus, one concerned with the body, or the somatic factor, and the other with the flagella—the flagellar element; Beyer and Reagh (1904) showed that these two factors could be further differentiated by the action of heat. Felix (1924), in an investigation of *B. typhosus*, claimed that there was a definite relation between the presence of somatic agglutinin in the serum and recovery from disease. Arkwright (1927), investigating "enteric" vaccines, demonstrated that the "H" or flagellar antigen had little or no value in a prophylactic vaccine, and stated "that the heat-stable smooth 'O' agglutinogen is probably identical with the antigen which produces resistance and with the smooth heat-stable agglutinable substance." Thus it would be possible, by obtaining a solution rich in flagella and poor in cell bodies, to have a vaccine of high agglutinogenic properties but poor powers of creating protection in animals, and such is probably the nature of the centrifugate obtained from such organisms, many of the numerous flagella being washed off the bacilli in the course of preparation or possibly separated from dead bacilli. If these latter had also undergone autolysis, some of the somatic factor would be present. Since the *V. cholerae* is monoflagellate the case is somewhat different as the heat-labile factor is present to a comparatively slight degree. This fact has caused much doubt as to the existence of two antigenic components in this micro-organism; Brutsaert (1924) stated that the heat-stable element only was present, while Balteanu (1926) elaborated a method, by which he obtained a flagellar emulsion from a culture of *V. cholerae*. This necessitated prolonged shaking and centrifuging in order to separate the flagella from the bodies. An ordinary centrifugate of *V. cholerae* contains only a small amount of flagellar material and other products of autolysis, and

consequently has only a feeble agglutinogenic action. It has, moreover, very little protective power for animals.

CONCLUSION.

The substance of *V. cholerae*, which, on inoculation, gives rise to protection in animals, is intimately connected with the body protoplasm; it is also heat-stable (100° C. 1 hour). It is present only in small quantity in the supernatant fluid of a 24-hour bouillon culture after centrifugalization.

I should like to express my grateful thanks to Prof. Besredka, of the Pasteur Institute, Paris, in whose laboratory most of the work was performed, for his helpful advice; to Prof. J. C. G. Ledingham for much useful criticism; and to the Governing Body of the Lister Institute for the facilities to complete this investigation.

REFERENCES.

ARKWRIGHT, J. A.—(1927) *J. Path. & Bact.*, **30**, 345.
BALTEANU, I.—(1926) *Ibid.*, **29**, 251.
BESREDKA, A.—(1927) *Rev. d'hyg. et de méd. prévent.*, **49**, 445.
Idem, AND GOLOVANOFF, M.—(1923) *C.r. Soc. de Biol.*, **89**, 933.
BEYER, H. G., AND REAGH, A. L.—(1904) *J. Med. Res.*, **12**, 313.
BRUTSAERT, P.—(1924) *C.r. Soc. de Biol.*, **91**, 1157.
CANTACUZENE, J.—(1920) *Ann. de l'Inst. Pasteur*, **34**, 57.
CASTELLANI, A., AND MENDELSON, R W.—(1915) *Brit. Med. J.*, **2**, 711.
DOPTER, CH.—(1915) *Ann. d'hyg. publique*, **23**, 65.
FELIX, A.—(1924) *J. Immunol.*, **9**, 115.
FERRAN, J.—(1885) *C.r. Acad. des Sci.*, **100**, 958.
FERRY, N. S., AND FISHER, L. W.—(1924) *Brit. J. Exper. Pathol.*, **5**, 185, 205.
FOX, C. J.—(1916-17) *Ind. J. Med. Res.*, **4**, 335.
GLOTOFF, E.—(1923) *C.r. Soc. de Biol.*, **89**, 368.
HAFFKINE, W. M.—(1913) 'Protective Inoculation against Cholera,' Calcutta (Thacker, Spink & Co.).
HORDER, SIR T., AND FERRY, N. S.—(1926) *Brit. Med. J.*, **2**, 177.
LANDSTEINER, K., AND LEVINE, P.—(1927) *J. Exp. Med.*, **46**, 213.
LEVADITI, C., AND MUTERMILCH, S.—(1908) *C.r. Soc. de Biol.*, **94**, 844, 1151.
MIURA, Y.—(1920) *J. Immunol.*, **5**, 145.
PERLZWEIG, W. A., AND KEEFER, C. S.—(1925) *J. Exp. Med.*, **42**, 747.
PFEIFFER, R., AND ISSAEFF.—(1894) *Ztschr. f. Hyg.*, **17**, 355.
SMITH, T., AND REAGH, A. L.—(1903-4) *J. Med. Res.*, **10**, 89.
STRONG, R. P.—(1907) *Philippine J. Sci.*, **2**, 413.
TAKANO, R., OHTSUBO, I., AND INOUYE, L.—(1926) 'Studies of Cholera in Japan,' Geneva (League of Nations, Health Organization), p. 41.
VINCENT, H.—(1915) *Rev. d'hyg.*, **37**, 321.
WRIGHT, A. E., AND BRUCE, D.—(1893) *Brit. Med. J.*, **1**, 227.

THE SUITABILITY OF THE GUINEA-PIG FOR EXPERIMENTAL WORK WITH VACCINIA VIRUS.

J. O. W. BLAND, L.M.S.S.A.,

Freedom Research Scholar.

From the Hale and Dunn Clinical Laboratories, London Hospital.

Received for publication March 16th, 1928.

THE suitability of the guinea-pig for work with the virus of vaccinia seems to be questioned by those who have studied this virus. Gildemeister and Herzberg (1925) certainly refer to the fact that guinea-pigs inoculated in the plantar skin with vaccinia virus develop a local vesicle, but the general impression appears to be that the guinea-pig is very much inferior to the rabbit for vaccinia work, and there seems even to be some doubt whether vaccinial lesions can be produced on the epilated hairy skin of the guinea-pig.

During the last few months I have had the opportunity of making some observations on this point, and the results obtained do not support the generally accepted view. Three strains of vaccinia derived from calf-lymph have been inoculated upon the skin of guinea-pigs for a varying number of generations and frequent titrations of the virus have been made. Although it is not claimed that the susceptibility of the guinea-pig is equal to that of the rabbit, the findings indicate that the former is quite a useful animal for experimental work with this virus.

Technique of Inoculation and Preparation of Passage Material.

Areas of the skin of the flanks about 1 in. square are epilated. Four such areas are usually prepared, two on each flank, but should a fifth be necessary there is room for it on the middle of the back. The skin is cleaned with soap and water followed by alcohol and ether, and is then lightly scarified with a sterile needle. The dilution of vaccinia virus is dropped on to the prepared areas with a sterile pipette and lightly rubbed into the skin. In making titrations of a sample of virus dilutions in M/50 phosphate pH 7·6 —the diluent used throughout this work—are made with precautions to avoid carrying over. Approximately equal quantities of each dilution are applied to separate areas. During this procedure and the subsequent examination of the animals care must be taken in handling them not to convey material from one area or pig to another, and for the same reason pigs under experiment should be kept in separate cages. There is a real danger of errors creeping into the titrations if these precautions are not observed.

On the third day after inoculation small red papules appear. These papules rapidly increase in size, and by the fourth day have become definitely vesicular. In another twenty-four hours they are pustular, and by the sixth

day they are beginning to dry up. Healing is complete in about 14 days, and there is little or no scarring.

When lesions have developed to the point of vesicles or pustules the pig is killed and the inoculated area cleaned with alcohol and ether. Using sterile precautions throughout, the lesions are scraped with a scalpel, and the scrapings weighed and ground up in a mortar. Phosphate solution is added to make the required dilution, usually 1/50 or 1/100, and the material is pipetted off into a test-tube and allowed to sediment in the ice-chest. The supernatant fluid constitutes the virus. It is more or less turbid and sometimes contains fine particles, which centrifugation does not throw down, and which are removed, if necessary, by filtration through sterile filter-paper.

The virus is kept, without any preservative, in the ice-chest, and under these conditions retains its full virulence for at least 48 hours, and in some cases for a week or more. Virus so prepared is nearly always contaminated by staphylococci, but other contaminations have not been observed.

In the earlier experiments sand was used in grinding up the skin scrapings, but evidence having been obtained that sand adsorbs a considerable quantity of virus, its use has been discontinued, and the tissue fragments are ground up in a mortar with the phosphate diluent only.

The loss of virus by adsorbtion on the sand is made evident in the following experiment: The scrapings from vaccinial lesions were weighed, ground with sterile sand and diluted with M/50 phosphate to give a 1% suspension. After a brief sedimentation this suspension was divided into two parts:

(*a*) A portion of the more or less turbid supernatant fluid which was filtered once through sterile filter-paper.

(*b*) The remainder of the supernatant fluid together with the deposit of sand and tissue fragments. The whole of this was filtered through filter-paper, and the filtrate was refiltered four times through the sand which had collected on the paper.

The virus titration of these two fractions is shown in Table I:

TABLE I.—*Effect of Sand Filtration on a Virus.*

Material inoculated.	Dilutions of material.			
	1/1000.	1/5000.	1/10,000.	1/100,000.
Virus filtered once through filter-paper	+++	$+_2$	$+_3$	0
Virus filtered five times through paper and sand	$+_4$	0	0	0

+ + + = Confluent vesicles. 0 = No take. + = Discrete vesicles, the number indicated by the figure. Readings taken on sixth day after inoculation.

Results.

The results obtained with three strains, all derived from calf lymph, are given in Table II and require little further explanation.

Calf lymph is not potent for the guinea-pig in high dilutions. The end-point in titrations lies somewhere between 1/100 and 1/1000. Such a titre is low compared with that found in the rabbit. Lymph from the source from

TABLE II.—*Pedigrees of Three Strains of Vaccinia in the Guinea-pig.*

No. of generations in guinea-pig.	Vc. V.	Vc. III.	Vc. I.
1	Calf lymph 1/100	Calf lymph no titration	Calf lymph 1/100
2	1 1/10,000	1 1/1000	1 1/100
3	2 1/1000	3 1/100	2 no titration
4	3 no titration	4 no titration	3 1/10,000
5	4 1/100 ; 5 1/50	7 no titration	5 1/10,000
6	5 1/5000	8 1/100,000	8 1/10,000
7	6 1/100,000	8 no titration ; 17 no titration	9 1/10,000
8		10 1/1000 ; 18 1/10,000	12 1/100 ; 15 −1/100
9		11 1/10,000 ; 19 no titration	13 1/50
10		12 1/10,000 ; 20 1/100,000	14 −1/50
11		21 1/10,000	
12		22 1/100,000	

The figure above the line in each passage is the serial number of the subculture of virus. The bottom figures give the titre of the subculture. This is the highest dilution which produced any vesicles. A minus sign preceding a fraction = no take at this dilution.

which that used in my work was drawn, Ledingham (1924) found potent in the rabbit by intradermal inoculation in a dilution of 1/10,000, and Gordon (1925), who made a number of titrations by scarification in that animal, obtained end-points between 1/1000 and 1/100,000 for samples drawn from different sources.

The guinea-pig, therefore, is not suitable for the titration of calf lymph, but study of Table II shows that high titres are obtained when the virus has become adapted to the species. It would appear that the strain Vc. I adapted itself to propagation in the skin of the guinea-pig with readiness, and that it now possesses a more or less fixed virulence for this animal species. The other two strains, Vc. III and Vc. V, though taking fairly well in the guinea-pig,

have shown more fluctuation in titre than did Vc. I, and it remains to be seen whether after further passage they will become as satisfactory as Vc. I.

Irregularities of this kind and the sudden collapse of Vc. I at the seventh generation are phenomena occasionally met with in work with viruses, and there seems no reason to ascribe them to the species of animal used.

In order to ensure that the lesions produced in the guinea-pigs were due to vaccinia and not caused by a virus present in the guinea-pig skin, which had been rendered virulent by passage, a neutralization experiment was carried out with the sixth generation of Vc. I and a potent anti-vaccinia serum. The result of this experiment, given below (Table III), leaves no doubt that one was dealing with the vaccinia virus:

TABLE III.

Mixtures of equal parts of virus and serum.			Result of inoculation in the guinea-pig.
Virus 1/500 + anti-vaccinia serum 1/2	Stood at room temperature 1 hour		—
Virus 1/500 + normal rabbit serum 1/2	Ditto		+++
Virus 1/500 + M/50 phosphate	,,		+++

CONCLUSIONS.

1. The guinea-pig is susceptible to vaccinia virus (calf lymph), though less so than the rabbit.

2. When inoculated on the epilated hairy skin with vaccinia virus by scarification, the guinea-pig reacts in a typical manner.

3. Vaccinia virus (calf lymph) can be readily adapted to the guinea-pig by passage and will then show titres little inferior to those obtained in the rabbit.

I wish to record my indebtedness and thanks to Dr. Bedson for the advice and help which he has so often given me.

REFERENCES.

GILDEMEISTER, E., AND HERZBERG, K.—(1925) *Deutsche med. Wchnschr.*, **51**, 1047.

GORDON, M. H.—(1925) *Med. Res. Council; Spec. Report Series*, No. 98. London (H.M. Stationery Office).

LEDINGHAM, J. C. G.—(1924) *Brit. J. Exper. Pathol.*, **5**, 332.

EXPERIMENTS ON THE VIRUS OF FOWL PLAGUE. (II)

CHARLES TODD.

From the National Institute for Medical Research, Hampstead, London.

Received for publication March 31st, 1928.

THE PRODUCTION OF ACTIVE IMMUNITY TO FOWL PLAGUE.

IT is now generally recognized that, in countries which have been free from outbreaks of fowl plague for some years the domestic fowl is invariably susceptible to the disease, and that artificial inoculation with the virus always gives rise to a fatal attack.

The few apparent exceptions to this rule are probably to be attributed to immunity subsequent on a naturally contracted attack of the disease, as it is known, since the work of Ostertag and Wolfhügel (1902), and more recently that of Erdmann (1920), that in epidemics of fowl plague the naturally occurring disease is not always fatal, but that a certain number of fowls, particularly young birds, may not succumb; and that such recovered fowls subsequently show an absolute immunity, so that in districts where the disease is endemic immune birds may be met with.

The knowledge that the fowl is capable of naturally acquiring such a high degree of immunity has led to numerous attempts to produce this by artificial means, but, although the problem has been attacked by many workers, the results have been most disappointing. Attempts to immunize fowls with heated virulent materials (blood and organs) made by Maggiora and Valenti, Maue (1904), Russ (1906), v. Prowazek (1908) and others were uniformly unsuccessful, and in 1907 Kraus and Schiffmann stated that a review of the literature showed that at that date no worker had been definitely able either to produce immunity in susceptible animals or to demonstrate the presence of antibodies—with the possible exception of Maggiora and Valenti (1904), who claimed to have produced a protective serum by the inoculation of the adult goose, which is naturally immune to the virus except when inoculated intracerebrally.

All attempts to produce an active immunity by the injection of the virus modified by the action of heat, light or other agents having given entirely negative results, the problem was next attacked on the lines of Pasteur's method for the immunization of animals against rabies, and in 1907 Kraus and Schiffmann found that it was possible to immunize young geese, which are less susceptible than fowls, by the injection of the dried spinal cord of infected young geese. Later in 1915 Kraus and Loewy were able to produce immunity in fowls by the same method.

In 1920 Jouan and Staub described the successful vaccination of fowls with heated virulent blood. These workers used virulent blood which had been heated for 3 days at 46–47° C., or for three-quarters of an hour at 60° C., and obtained equally good results in either case. The heated blood was injected intramuscularly and one injection was sufficient to produce a solid immunity,

but it was necessary to use a comparatively large volume of the heated blood —not less than 10 c.c.

In view of the quite unanimous experience of all previous workers that immunity could not be produced by the injection of heated virulent materials —whether blood or organs—Jouan and Staub's results are of great interest, and it is not easy to see how they are to be explained. It is true that they gave a large dose of the virulent blood, but immunity was produced after a single dose, whereas all other workers have failed to obtain immunity even after repeated injections of the heated virus, and since the publication of Jouan and Staub's work, Doyle (1927), who treated 4 fowls with a series of 6 injections of 1 c.c. of an emulsion of the spleen of infected fowls—this emulsion having been heated at 60° C. for an hour—did not obtain immunity in any of the birds.

Gerlach and Michalka (1926) also were unable to immunize birds with large and repeated doses of the dead virus.

In 1926 Staub reported that he was able to vaccinate fowls by means of injections of a formalinized saline emulsion of the spleen of infected birds, this vaccine being prepared by emulsifying 1 gm. of the spleen with 5 c.c. of a 1 in 500 solution of formalin in saline, and keeping the mixture at 37° C. for 48 hours. A single dose of the vaccine was sufficient to immunize a fowl. Doyle (1927) repeated these experiments with formalinized emulsions, but was unable to obtain immunity against a subsequent dose of virulent blood, even 6 progressively increasing doses of the formalinized vaccine—from 1 c.c. to 5 c.c.—given at 5-day intervals not producing immunity.

In view of this unsatisfactory state of affairs it was decided to make further attempts to find a reliable method of immunization, and for the experiments, except where otherwise stated, the strain of the virus used was one obtained from an outbreak of the disease in England (Rice, 1922).

Attempts to Immunize with the Heated Virus.

In the course of an outbreak of fowl plague in Egypt some years ago the writer made repeated attempts to vaccinate fowls with heated virulent materials (blood, spleen, etc.). These experiments gave uniformly negative results, so that there was very little inducement to repeat them, but in view of Jouan and Staub's apparent success and of the possibility that the particular strain of the virus used might influence the result, one or two further trials were made.

Two fowls were injected respectively with 8 c.c. and 15 c.c. of virulent blood (Staub's strain) which had been heated at 58° C. for 30 mins. 16 days later these birds were given a small dose of the unheated virus, and both died of fowl plague in less than 48 hours.

Similarly a fowl injected with 15 c.c. of virulent blood (English virus), heated in the same manner, showed no immunity.

Immunization with Formalinized Virus.

For these experiments the liver of a fowl which had died from the disease was ground up in a mortar with glass powder and emulsified with five times its weight of M/200 phosphate saline (pH = 7·2), to which 1 part in 500 of commercial formalin had been added. The emulsion was filtered through

absorbent cotton-wool and kept in the incubator at 37° C. for 48 hours, after which it was placed in the cold room. Three fowls received varying doses of the vaccine, and 12 days after the last dose were tested with a small dose of the virus:

Fowls each receiving One Dose of Formalinized Virus.

Serial number.	Dose of vaccine.	Tested after—	Result.
104	2 c.c.	12 days	Died in 41 hours.
106	5 „	„	„ „
108	10 „	„	„ 3½ days.

As even the largest dose (viz. 10 c.c.) had not conferred more than a suggestion of immunity a second experiment was made with the same vaccine, but in this case each fowl was given three injections at intervals of a week:

Fowls each receiving Three Doses of Formalinized Vaccine.

Serial number.	Dose of vaccine.	Tested after—	Result.
103	2 c.c.	9 days	Died in 6 days.
105	5 „	„	Died in 3 days (found to be infected with avian tubercle).
107	10 „	„	Died in 9 days.

Thus in the case of these birds which had received three injections death took place after a longer interval, but, even in the case of the bird receiving three doses of 10 c.c. of the vaccine, subsequent injection of the virus resulted in a fatal attack of the disease with very pronounced lesions, and a normal fowl injected with ½ c.c. of an emulsion of the liver of the dead bird died in 45 hours, showing that the virulence of the virus was in no way modified.

Further experiments with the formalinized virus, prepared both from organs and from virulent blood, yielded similar results, and it was decided that the method was not sufficiently promising to merit further trial.

Immunization with Phenol Glycerine Vaccine.

As the virus of fowl plague in several respects resembles that of rabies, the work of Fermi (1908) and of Umeno and Doi (1921) on the use of phenolized vaccines suggested their trial in the case of fowl plague. Accordingly, the liver of a fowl which had died of the disease was ground up in a mortar with glass powder, and emulsified with four times its weight of the following solution:

Phenol	0·5 gm.
Glycerine	60·0 c.c.
Distilled water	39·5 „

The resulting emulsion was placed in a stoppered bottle and kept in a dark cupboard in the laboratory at a temperature of approximately 20° C. After remaining for seven days at this temperature the vaccine was ready for use and was transferred to the cold room.

Six normal fowls were treated with the vaccine so prepared, each bird

receiving three doses at intervals of a week. In the case of four of the fowls the doses were $\frac{1}{2}$, 1 and 3 c.c., in the fifth $\frac{1}{2}$, 1 and $1\frac{1}{2}$ c.c., and in the sixth $\frac{1}{2}$, 1 and 2 c.c.

Approximately a week after the last dose of the vaccine each of the birds received an injection of $\frac{1}{3}$ c.c. of a virulent blood, which killed a normal fowl at a dose of a millionth of a cubic centimetre.

None of the vaccinated fowls showed any symptoms, and they were all subsequently inoculated with 5 c.c. each of a highly virulent blood without noticeable effect.

The vaccine is thus very effective, as after three comparatively small doses all the birds had acquired a high degree of immunity—showing no symptoms on the subsequent injection of 300,000 times the amount of virus required to kill a normal fowl. Three doses of the virus, however, appear to be required to ensure a certain immunity.

A number of fowls have since been immunized in the same way and so far only one failure has been met with. In this case the vaccine had been hastily prepared and the liver tissue was very imperfectly emulsified. In this connection one or two details concerning the preparation of the vaccine may not be out of place. In fowls infected with fowl plague the concentration of the virus in the blood varies within somewhat wide limits in different individual birds, and it seems not improbable that this may also be the case as regards the concentration of the virus in the liver. It is therefore advisable to use the livers of several fowls for the preparation of a batch of the vaccine.

In the absence of a grinding machine the liver-tissue was ground with glass powder in a mortar. This was effected quickly and efficiently by the use of short lengths of glass tubing which had been heated in the blowpipe and chilled by dropping into sterilized tap-water. Glass so treated breaks down easily in the mortar, and yields a mass of sharp-edged particles which are much more effective than sand: on allowing the vaccine to stand the larger fragments of glass settle to the bottom, and any smaller particles which may be present in the supernatant fluid do not appear to give rise to trouble in the injected birds.

After grinding the liver and emulsifying it with the phenol-glycerine solution the emulsion was filtered through a wire sieve so as to eliminate any larger particles or shreds of tissue which might block a medium-sized hypodermic needle, and was kept in the dark at 20° C. in a stoppered glass bottle which was shaken from time to time.

A number of batches of the vaccine were tested daily by intramuscular injection into normal fowls. The individual vaccines showed slight differences in their behaviour, probably dependent on the degree of fineness to which the tissue had been ground and also on the amount of blood present in the liver, but as a rule it was found that the injection of $\frac{1}{2}$ c.c. of the vaccine no longer gave rise to fowl plague after it had been kept for about 5 days at 20° C. In three cases the vaccine was non-virulent after 4 days and in two cases after 5 days. In no case did a vaccine which had been kept as above for 7 days give rise to the disease.

After 7 days at a temperature of 20° C. the vaccine was placed in the cold room at — 3° C. (approximately), where it was kept until required for use.

The period for which it retains its efficacy at this temperature has not been adequately examined, but it seems that deterioration takes place somewhat rapidly, as three different vaccines tested after keeping for 70, 86 and 120 days respectively at — 3° C. failed to vaccinate fowls receiving the usual three doses of 1·0, 2·0 and 3·0 c.c. given at intervals of a week.

As the cellular layer of centrifuged virulent blood contains the virus in a high concentration, it was thought that these cells might constitute a better material for the manufacture of a vaccine than the liver-tissue—being already in a condition of uniform emulsion. A vaccine was accordingly prepared exactly as before, except that the liver-tissue was replaced by an equal weight of the washed corpuscles from virulent blood, and naturally grinding was not necessary. The vaccine was kept at 20° C. and tested at frequent intervals. No loss of virulence, however, occurred, and even after 53 days, when the tests were discontinued, the mixture was apparently as virulent as when first prepared. It would seem that either the hæmoglobin or some other constituent of the blood-cells prevents the action of the phenol on the virus, so that, in preparing a vaccine from the liver, it is desirable to avoid the presence of any large amount of blood by first roughly mincing the organ, and then washing away any blood-clots with saline before the final grinding.

Comparison of Various Strains of the Virus.

Being in possession of a satisfactory method of producing immunity to the disease, it was interesting to see how different strains of the virus would behave in cross-immunity experiments, and fowls immunized with the English strain were tested by inoculation with the virulent blood of fowls infected with the three following strains of the disease which were available in the laboratory—Dutch (de Bliech), French (Staub), and German (Miessner's goose virus). In no case did the birds show any symptoms, so that these strains would appear to be at any rate closely related. The reverse experiment has not yet been carried out.

Immunization with Tricresol Glycerine Vaccine.

In order to see if the higher phenols could be used in the same way as carbolic acid for the preparation of a vaccine a few experiments were made with tricresol. The liver of a fowl which had died of fowl plague was divided into three parts, each of which was used for the preparation of an emulsion, the technique being as in the case of the phenol glycerine vaccine, except that in the fluid used for preparing the emulsions the phenol was replaced by tricresol—0·1 %, 0·2 % and 0·5 % of tricresol being used respectively in the three cases. Tested after 8 days at 20° C. the first emulsion was found to be still virulent and was discarded. The second and third were used for the immunization of fowls, and after three doses (0·5 c.c., 1·0 c.c. and 2·0 c.c.) at intervals of a week these birds were found to have a solid immunity.

It would thus appear that the tricresol glycerine vaccine is at least as effective as the phenol glycerine vaccine.

Although the form of vaccination described above gives rise to a solid immunity, it is hardly necessary to state that there is no suggestion of putting

it forward as a method of dealing with outbreaks of the disease—where much more radical measures are called for. It is perhaps rather in connection with certain other diseases due to ultramicroscopic viruses that the data obtained might find useful application, and the efficacy of a phenol glycerine vaccine in the case of fowl plague—a disease so notoriously difficult to immunize against—suggests the trial of a similar vaccine in such diseases as cattle plague and swine fever, the viruses of both of which show a striking resemblance in their behaviour to the virus of fowl plague.

CONCLUSIONS.

1. Attempts to immunize fowls against fowl plague with heated virulent materials (blood, spleen, etc.) gave entirely negative results.

2. Attempts to immunize with the formalinized virus were also unsuccessful.

3. A solid immunity against the disease can be constantly produced in fowls by the use of a phenol glycerine vaccine prepared from the liver of birds dead of the disease.

4. In order to ensure immunity three doses of the phenol glycerine vaccine, given at intervals of approximately a week, appear to be required.

5. An apparently equally satisfactory vaccine can be prepared by the use of tricresol in place of phenol in the above vaccine.

6. Fowls immunized with three small doses of the vaccine show no noticeable symptoms on the subsequent injection of a dose of the virus at least 300,000 times that required to kill a normal fowl.

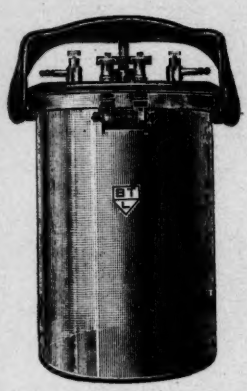
7. Birds immunized with a vaccine prepared from the English strain of the virus were found to be immune against the Dutch (de Bliech), the French (Staub) and the German (Miessner—goose) viruses.

The writer is indebted to Mr. F. M. Doyle, M.R.C.V.S., for the Dutch and the French strains of the virus, and to Prof. Dr. Miessner, of Hanover, for the strain isolated by him from the goose.

REFERENCES.

Doyle, F. M.—(1927) *J. Comp. Path.*, **40**, 144.
Erdmann, R.—(1920) *Arch. f. Protistenk.*, **41**, 190.
Fermi, C.—(1908) *Ztschr. f. Hyg.*, **58**, 233.
Gerlach, F., and Michalka, J.—(1926) *Deutsch. tierärtz. Wchnschr.*, **34**, 897.
Jouan, C., and Staub, A.—(1920) *Ann. de l'Inst. Pasteur*, **34**, 343.
Kraus, R., and Schiffmann, J.—(1907) *Cntrlbl. f. Bakt.*, **43**, 825.
Kraus, R., and Loewy, O.—(1915) *Ibid.*, **76**, 343.
Maggiora, A., and Valenti, G. L.—(1913) Cited by von Ostertag, Kolle and Wassermann's *Handbuch*, **6**, 280.
Idem.—(1904) *Ztschr. f. Hyg.*, **48**, 280.
Maue.—(1904) *Arb. a. d. Kais. Gesundheitsamt.*, **21**, 537.
Ostertag and Wolfhügel.—(1902) *Monatsschr. f. prakt. Tierheilk.*, **14**, 49.
von Prowazek, S.—(1908) *München. med. Wchnschr.*, **55**, 165.
Rice, J. P.—(1922) *Vet. Record*, **4**, 207.
Russ, V. K.—(1906) *Arch. f. Hyg.*, **59**, 286.
Todd, C.—(1928) *Brit. J. Exper. Pathol.*, **9**, 19.
Umeno, S., and Doi, Y.—(1921) *Kitasato Arch. Exp. Med.*, **4**, 89.

BAIRD & TATLOCK (LONDON) LTD



An Improved Form of

ANAËROBIC JAR

By Drs. McINTOSH & FILDES.

(*Reference: 'British Journal of Experimental Pathology,' 1921, II, 153.*)

B. 1488. **Price £3 15s. each**

Sole Address:

14-15 CROSS STREET HATTON GARDEN LONDON EC1

Subscription in India and abroad Rs. 16 per annum. Post free. Payable in advance.

— THE —

INDIAN JOURNAL OF MEDICAL RESEARCH

Edited by THE DIRECTOR-GENERAL, INDIAN MEDICAL SERVICE, THE PUBLIC HEALTH COMMISSIONER WITH THE GOVT. OF INDIA and THE DIRECTOR OF MEDICAL RESEARCH in collaboration with the leading authorities on Tropical Medicine.

The Indian Journal of Medical Research is published quarterly. Each number contains about 200 pages and is Illustrated with Plates in Colour and Half-tone and with Diagrams and Charts, and is the only medium publishing the results of the best medical research work being carried on by leading scientific medical men in India and the East.

THACKER, SPINK & CO., P.O. Box 54, CALCUTTA.

RATES FOR ADVERTISEMENTS.

	One insertion	Six (each)	Twelve (each)
	£ s. d.	£ s. d.	£ s. d.
Whole page	4 10 0	4 5 0	4 0 0
Half page	2 10 0	2 5 0	2 2 6
Quarter page	1 7 6	1 5 0	1 2 6

All communications respecting Advertisements in this Journal should be addressed to the ADVERTISEMENT MANAGER, 27, Southampton Street, London, W.C. 2. Telephone: Gerrard 0693.

THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY.

VOL. IX, No. 2. APRIL, 1928.

CONTENTS.

SMITH, J.—THE MODIFICATION OF SCARLATINAL TOXIN BY FORMALDEHYDE 49

CRAGIE, JAMES.—A METHOD OF STAINING BACTERIAL FLAGELLA . . . 55

HERBERT, FREDA K.—PANCREATIC FAT NECROSIS: A CHEMICAL STUDY . 57

PARKES, A. S., AND DRUMMOND, J. C.—THE RELATION BETWEEN FATNESS AND STERILITY 63

KERRIN, JOHN C.—THE INCIDENCE OF *B. TETANI* IN HUMAN FÆCES . . 69

BYROM, F. B., AND KAY, H. D.—BLOOD PHOSPHORUS IN HEALTH AND DISEASE: III.—A. THE ALLEGED VALUE OF BLOOD PHOSPHORUS DETERMINATIONS IN SUSPECTED MALIGNANT DISEASE.—B. THE PHOSPHORUS DISTRIBUTION IN ANÆMIA, POLYCYTHÆMIA, AND LEUKÆMIA 72

SMIRK, FREDERICK HORACE.—I. THE ACCURATE MEASUREMENT OF THE PROPORTION OF CORPUSCLES AND SERUM IN BLOOD.—II. THE VOLUME OF RED BLOOD-CORPUSCLES IN VENOUS AND OXYGENATED BLOOD AND AFTER EXPOSURE TO VARIOUS SATURATIONS OF CARBON DIOXIDE . 81

FAIRBROTHER, R. W.—THE STRUCTURE OF THE *V. CHOLERÆ*, WITH REFERENCE TO ITS IMMUNIZING PROPERTIES 89

BLAND, J. O. W.—THE SUITABILITY OF THE GUINEA-PIG FOR EXPERIMENTAL WORK WITH VACCINIA VIRUS 97

TODD, CHARLES.—EXPERIMENTS ON THE VIRUS OF FOWL-PLAGUE. (II) . 101

Subscriptions and Business Correspondence should be addressed to the Publishers, 28, Gower Place, London, W.C. 1. The JOURNAL is only sold *at the subscription rate* by the year or volume. General correspondence for the Editors should be addressed to Dr. P. FILDES, London Hospital, E. 1, but letters relating to biochemical and physiological papers to Professor E. C. DODDS, Middlesex Hospital, W. 1.

PRINTED BY ADLARD AND SON, LIMITED, LONDON AND DORKING.